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Conservation and Transformation of Energy by Bacterial Membranes

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"Disconcertingly few laymen—even few college graduates—really understand what the scholar means by 'truth.' It is not a citadel of certainty to be defended against error; it is a shady spot where one eats lunch before tramping on. The professional thinker enjoys being where he is, but he also looks forward to new vistas around the next bend, over the next crest."—Lynn White, Machina ex Deo.

INTRODUCTION

Most of the triumps of biochemistry were won by reducing the exquisite architecture of the living cell to a homogenate. Now that the electron microscope has revealed the sophistication of cellular structure, it seems astonishing that our brutal methods should have been able to generate those intricate charts of metabolic pathways which adorn laboratory walls: catabolism, biosynthesis, even transcription and translation have been successfully analyzed by assuming that, for practical purposes, a cell is just a bag of enzymes. Processes associated with membranes—oxidative phosphorylation, transport, motility and cell division—have been more refractory, but one might well attribute this merely to the tech-

nical difficulties of studying largely insoluble proteins.

The theme that pervades the present article is that membrane functions must be approached from a fundamentally different point of view. It seems evident that in bacteria, as in mitochondria and other organelles, certain enzyme systems are not just anchored to the cytoplasmic membrane but are organized within and even across the osmotic barrier. Systems of this kind catalyze metabolic reactions that are oriented with respect to the membrane: *vectorial* metabolism. By virtue of the spatial organization of the catalysts, chemical reactions of this kind may be accompanied by the translocation of molecules or groups across the membrane.

The enzymes with which we are most familiar are those that are soluble, and consequently catalyze reactions that have no macroscopic direction in space. However, at the level of each individual enzyme molecule, many if not all enzymic processes must be thought of as having a particular orientation relative to the active site. Enzyme complexes, such as those which carry out the oxidation of pyruvate or of fatty acids (not to mention ribosomes), clearly depend upon the precise articulation of successive molecular events. But only in the case of reactions which take place across a membrane would the implications of vectorial metabolism be fully apparent, since such reactions result in mass translocation from one side of a barrier to the other: So long as the barrier is intact, vectorial reactions may generate differences in concentration across the barrier and, if ions or electrons are translocated, differences in electrical potential. Conversely, the rate and extent of a vectorial reaction will be influenced by the concentration of reactants in the two compartments and possibly by the potential difference. Finally, two reactions can be coupled through gradients of concentration or of electrical potential, so as to make one reaction drive the other even though they do not share a common intermediate (chemiosmotic coupling). Since vectorial metabolism would require precise orientation of the components, structure and function become inextricably intertwined.

Do vectorial reactions across membranes exist in fact as well as in theory? Although the concept is only now receiving widespread attention, it is by no means a novel one. More than forty years ago, Lund suggested that bioelectric phenomena may result from the separation of charges during the redox reactions of respiration. Lundegardh subsequently proposed specifically that reduction of a cytochrome by a hydrogen carrier, such as flavine, results in the release of a proton on one side of a membrane. This was the germ of the idea that the catalysts of the respiratory chain are so oriented as to separate protons and electrons across a membrane, and that many fundamental membrane phenomena ultimately depend upon this separation of electrical charges. Robertson (317) has recently surveyed the genesis and subsequent evolution of this idea, from which sprang such diverse concepts as Conway's redox hypothesis of active transport, efforts to understand acid secretion by the stomach, and Mitchell's chemiosmotic hypothesis of oxidative phosphorylation. Research over the past decade has made it plausible, if not certain, that several enzyme systems built into biological membranes do catalyze vectorial metabolism. Among these are the oxidation chains of respiration and photosynthesis, adenosine triphosphatase (ATPase) complexes which translocate Na^+ and K^+ across mammalian cell membranes and perhaps protons across the membranes of mitochondria and bacteria, the phosphotransferase system for sugar uptake by bacteria, and others, less familiar.

The object of the present essay is to examine the role of vectorial processes in the generation of biological energy and its utilization by bacterial membranes: oxidative phosphorylation and photophosphorylation on the one hand, active transport and other work functions on the other. The principles that underlie these processes are presently more thoroughly understood in mitochondria and chloroplasts than they are in bacteria, and a major aim of this survey is to integrate knowledge from eukaryotic organelles with the more fragmentary data from prokaryotic cells. But insight flows both ways, and it is obvious that microorganisms have much to offer the student of basic membrane physiology: the capacity for growth both under aerobic and anaerobic conditions, in environments that are extreme with respect to *p*H, temperature, or ionic composition, and finally the powerful technique of specific mutations. It may be true that in molecular genetics bacteria have had their day in the sun, but in membrane physiology it is not yet noon.

Like all reviews of the literature, this one draws on the work of others for both insight and information. Not always do the references reflect this adequately since, in order to keep the total within reasonable bounds, I have elected to cite recent papers whenever possible, sometimes to the neglect of earlier pioneering work. Coverage extends to 1972. But I have leaned most heavily on the writings of

Peter Mitchell (256–259, 261, 264, 265). More than any other contemporary investigator, Mitchell has explored the experimental and theoretical implications of the idea that biochemical processes may be oriented in space. To him is also due much of the terminology employed to describe such processes, including the term vectorial metabolism. The extent of my indebtedness will be obvious to the reader.

A NOTE ON TERMINOLOGY

More than a little of the complexity of membrane physiology is semantic in origin, but not therefore trivial: the multiplicity of terms encountered in the literature reflects the divergent viewpoints of several schools of thought.

There is little difficulty over the first group of terms. *Uptake* is usually employed in a purely operational sense to designate removal of the substrate from the medium. The term covers translocation and adsorption, the substrate may or may not be chemically altered and nothing is implied regarding mechanisms. *Transport* and *translocation* refer in a general sense to any movement from one side of a membrane to the other. The mechanism is not specified, but in practice "transport" implies a process more complex than simple diffusion. *Facilitated diffusion* is distinguished from simple diffusion by signs pointing to interaction of the substrate with a component of the membrane; saturation kinetics and stereospecificity are observed, and the rate is greater than would be expected for passive diffusion across a lipid phase. However, by definition the process involves no energy input and results only in equilibration of the substrate across the membrane in accord with the electrochemical potential.

Difficulties begin to arise when we come to "carrier"-mediated transport that results in the accumulation of nutrients and metabolites against a concentration gradient. *Group translocation* is now generally used to describe the result of a reaction catalyzed by an enzyme system oriented across a membrane: chemical modification of the substrate occurs concurrently with its translocation (256, 258, 259, 321). Examples include the translocation of protons and electrons as well as the vectorial phosphorylation of sugars. Frequently, however, no chemical change of the substrate is demonstrable, and accumulation is attributed to *active transport*. Readers interested in the origin and precise definitions of this term must refer elsewhere (224, 259, 340, 358). Suffice it to mention here that the word is currently employed in two distinct senses. Rosenberg originally defined active transport as a process which results in the movement of a substance from a region of lower to one of higher electrochemical potential; movement against the electrochemical potential gradient requires input of "energy," and the mechanism of energy coupling can thus be seen to lie at the heart of the concept of active transport. Dependence upon metabolism is the most visible hallmark of transport processes labeled "active," but the actual linkage between translocation and the metabolic machinery can occur by diverse molecular mechanisms. Some are exceedingly indirect, as in the accumulation of sugars by the mammalian intestine at the expense of a sodium gradient which is in turn maintained by a specialized ATPase. For this reason, some investigators prefer the definition of Kedem who would restrict the usage of active transport to those translocations which, like the Na^+, K^+-ATPase, are *directly* linked to metabolic reactions.

Mitchell's terminology starts from the concept of vectorial metabolism (259); it avoids the ambiguities inherent in the concepts of "active transport" and "energy coupling," and will be employed in this article whenever possible. *Primary translocations* are those in which translocation is directly linked to a biochemical reaction, and may be of two kinds. *Group translocation*, defined above, occurs at the substrate level. *Enzyme-linked solute translocation* is a process in which the substrate itself does not participate in the exchange of covalent bonds, but is translocated as a result of such a reaction. This corresponds to Kedem's definition of active transport, and is exemplified by the Na^+, K^+, and the Ca^{2+} transport ATPases of mammalian cells.

Secondary translocations are by definition not directly linked to a chemical or metabolic reaction, but may be secondarily coupled. The simplest is *uniport*, the translocation of a single substrate by the carrier center, which corresponds to "facilitated diffusion." Uniport results in equilibration of the substrate across the membrane, in accord with its electrochemical potential. More complex situations arise when two substrates interact with the carrier (259). "Symport (cotransport) reactions are those in which two solutes equilibrate across an osmotic barrier such that the translocation of one solute is coupled to the translocation of the other in the same direction." In this event, an electrochemical gradient which impels the movement of one substrate (Na^+ or H^+, for example) can drive the movement of another substrate which rides on the same carrier

(sugar, or an amino acid), even though the latter may move against its own electrochemical potential. "*Antiport* (counter-transport) reactions are those in which two solutes equilibrate across a barrier such that translocation of one solute is coupled to the translocation of the other in the opposite direction." Thus, an asymmetrical distribution of one substrate will drive the movement of the other in the opposite direction. It is important to recognize that these are not merely hypothetical situations; uniport and antiport, at least, are established modes of action of ion-conducting antibiotics.

Concentrative transport is a convenient term to denote the familiar and ubiquitous capacity of living things to move metabolites or nutrients against apparent concentration gradients, without specifying any mechanisms.

The membrane components which mediate any of the above translocations will be referred to as "transport systems," implying nothing as to their number, nature of mode of action. The term porter is employed by Mitchell (258, 259, 264); strictly speaking, it applies only to the catalysts of secondary translocations. "Permease" still has adherents among microbiologists, but the meaning of the word has become cloudy: some investigators use it to designate the transport system as a whole, others refer specifically to that element which recognizes the substrates (see references 206, 207, 259, 301). These ambiguities render the term unsuitable for the present essay. The meaning of *carrier* and *carrier center* is self evident, even though their nature may be far different.

Finally, "energy" will be employed in the loose manner customary among biochemists to designate the capacity to do work.

ENERGY TRANSDUCTIONS IN MITOCHONDRIA

It is no longer heretical, or even novel, to suggest that mitochondria and chloroplasts evolved from microbial symbionts. Their probable microbial ancestry would in itself justify a section on organelles in a review of bacterial membranes, but there is a more compelling reason: Current concepts of the role of membranes in energy metabolism were developed and refined largely by studies with mitochondria and chloroplasts. The relatively detailed examination of mitochondria which follows is intended to help narrow the gap between the subcultures and to provide a point of departure for the discussion of bacteria.

Theories of Energy Conservation

The oxidation of reduced nicotinamide adenine dinucleotide (NADH), succinate, and other electron donors is catalyzed by a multienzyme chain associated with the inner (cristae) membrane of the mitochondrion. The oxidation chain, to use Racker's noncommital term (310), includes both hydrogen carriers (flavoproteins, quinones) and electron carriers (cytochromes). As a result of the oxidation, adenosine triphosphate (ATP) is synthesized with what appears to be a fixed stoichiometry of 3 moles of ATP per mole of NADH, 2 per mole of succinate. The coupling of ATP synthesis to the redox reactions takes place at specific sites in the chain; it involves an assembly of proteins, one of which has ATPase activity, which are collectively referred to as the coupling device.

One thinks of the respiratory chain primarily as a device for the synthesis of ATP, but in fact mitochondria carry out a variety of energy dependent processes: reversal of the direction of oxidation, as in the reduction of nicotinamide adenine dinucleotide (NAD) by succinate in the presence of ATP; transhydrogenation, the reduction of NAD by reduced nicotinamide adenine dinucleotide phosphate (NADHP); and, of particular concern in this article, the accumulation of ions and substrates against the concentration gradient. Oxidative phosphorylation proper, i.e., the production of ATP, is thus but one of several modes in which respiratory energy may be utilized.

Energy-linked functions can in general be supported either by oxidation of an electron donor or (with the oxidation chain blocked by lack of substrate or with inhibitors) by exogenous ATP. Hydrolysis of ATP has been shown to support ion accumulation and transhydrogenation, as well as reversed electron transport. The three coupling sites of the oxidation chain appear to be equivalent, and energy made available at one site can be used at another. Finally, ATP synthesis and other functions can also be driven by a preexisting ion gradient in the absence of other sources of energy. There thus appears to be a network of reversible reactions linking the redox chain, ATP, and ion concentration gradients (reviews: 42, 125, 150, 230, 308, 310, 350–352, 377)

The network was mapped chiefly by means of inhibitors of the "energy-transfer" reactions which result in the ultimate synthesis of ATP. Oligomycin, rutamycin, and dicyclohexylcarbodiimide (DCCD) inhibit both ATP synthesis and the ATPase activity of mitochondria and of submitochondrial particles. These inhibitors also block transhydrogenation and ion transport supported by ATP, yet do not

affect these reactions when an oxidizable substrate serves as energy donor (references to the original literature will be found in the reviews listed above). These findings are of the utmost importance for the argument developed here: They point to the existence of an energized entity or state, which can be generated at the expense of oxidation, ATP, or even of ion gradients and is the link between the various energy-dependent functions. Basically similar conclusions were reached from studies on submitochondrial particles depleted of ATPase; these can use oxidation but not ATP to support ion transport (350). The central position of this entity, variously referred to as "common factor" (125), "energy pressure" (352), or even plain "~" so as not to prejudge the thorny issue of its nature, is diagrammatically shown in Fig. 1.

Respiration and phosphorylation in mitochondria are normally coupled so that anything that retards phosphorylation retards respiration as well: lack of adenosine diphosphate (ADP), for example, or oligomycin. This is the well-known phenomenon called respiratory control. However, many conditions and reagents are known which dissociate the linkage: 2,4-dinitrophenol is only the best known of many "uncouplers" which allow respiration to proceed in the absence of phosphorylation. Many of these are believed to dissipate the energized common factor and block not only ATP synthesis but all the energy-linked functions.

Thus far there is little disagreement, particularly not about the crucial postulate of an energized state or entity preceding ATP. It is the nature and origin of this energy-conserving entity that is the bone of contention.

Chemical coupling hypothesis. The first hypothesis to be formulated explicitly (see Slater, 351, 352) has as its essential feature the postulate that the free energy released by the oxidation/reduction of adjacent electron carriers is conserved in the form of a high-energy intermediate, for instance:

$$A_{red} + B_{ox} + I \rightleftharpoons A_{ox} \sim I + B_{red}$$

This formulation is analogous to substrate-level phosphorylations such as the oxidation of 2-ketoglutarate, in which the energy released is conserved by formation of succinyl coenzyme A. Experimental findings which will not be recapitulated here made it necessary to postulate additional intermediates, including the nonphosphorylated intermediate X ~ I that serves as the common energy donor of Fig. 1 and a phosphorylated precursor of ATP, X ~ P.

There has been no lack of candidates for the chemical links between oxidation and phosphorylation, but thus far none have long withstood critical examination. This should perhaps not be surprising: intermediates may well be stable only in the hydrophobic environment of the lipid matrix in which the oxidation chain is embedded. This would surely be true for the high-energy forms of cytochromes which have recently been inferred from careful spectroscopic studies, especially in the region of the second coupling site (see 352, 395; but also 156). Storey (361) has formulated, in considerable chemical detail, a model in which redox energy is conserved as a strained S—S bond in a protein that participates in the redox reaction; energy transfer takes place by transesterification with an adjacent protein to form an acyl thioester, which in turn can interact with additional proteins. Again, such intermediates would not be expected to survive outside the membrane.

The failure to isolate intermediates of oxidative phosphorylation was the impetus that led to the formulation of alternative hypotheses. However, as we shall see below, the chemiosmotic and conformational hypotheses also invoke intermediates that have defied isolation. I would rather emphasize that, even in its contemporary form, the chemical hypothesis requires the membrane only to act as an organizer for the catalytic elements, and perhaps to supply a hydrophobic environment, but assigns to it no intrinsic function in energy-linked processes.

Conformational coupling. The most familiar transducer of biological energy is muscle, which converts the energy released by the hydrolysis of ATP into mechanical work; an analogous process, but operating in the reverse direction, might account for the synthesis of ATP. In this spirit, several investigators (27, 42, 124, 144, 293, 361, 403) have formulated

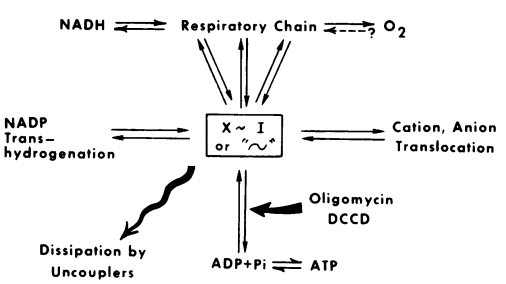

FIG. 1. *Pathways of energy conservation and transformation in mitochondria.*

hypotheses in which the energy released by the oxidation chain is conserved in the form of strained, metastable and energy-rich conformations of elements of the cristae membrane. In the presence of ADP and Pi, the energized structure would relax with concomitant formation of ATP. A mechanism has even been rediscovered (403)—albeit only in very schematic form—by which conformation changes could induce differences in *p*H and electrical potential across the membrane, and secondarily activate ion transport. Relatively little is presently known about high-energy conformations of proteins, and the conformational coupling hypothesis suffers from the paucity of both chemical detail and direct experimental support. So far as I am aware, the only pointed evidence comes from electron micrographs which illustrate drastic structural changes in mitochondria. The various energized and nonenergized forms are closely correlated with the metabolic state of the mitochondria. The difficulty remains of proving that the conformational changes are the cause, rather than the consequence, of metabolic events such as ATP synthesis or ion transport.

Formally, the conformational coupling hypothesis appears to be merely a variant of the chemical hypothesis, substituting a metastable structure for an unstable intermediate. But this statement unfairly belittles the significant difference in point of view. Green and his associates rightly stress the potential importance of this concept in unifying a wide variety of membrane processes. Unlike the chemical hypothesis, the conformational one assigns to the structure of the system a profound, albeit ill-defined role in phosphorylation and transport.

Chemiosmotic hypothesis. A distinguished colleague once described his attitude towards the chemiosmotic hypothesis as one of "admiring incomprehension," and it is my impression that his perplexity is widely shared in the microbiological community. This is a pity, for the controversy that was sparked by the introduction of the chemiosmotic hypothesis has transformed some of the basic premises of membrane research.

First formulated by Peter Mitchell in 1961 (255), the chemiosmotic hypothesis has been set forth in considerable detail. The intention of the present section is only to outline the argument, as simply as may be. For detailed and rigorous exposition readers must refer to articles by Mitchell (257, 261, 266) and especially to the lucid scrutiny of the hypothesis by the late G. D. Greville (125). The critical appraisals by Racker (310), Slater (352), and Skulachev (350) should also be consulted.

The chemiosmotic hypothesis rests upon the following basic postulates. (i) The inner mitochondrial membrane, in which the oxidation chain and coupling device are localized, is essentially impermeable to most ions, including both OH^- and H^+. In consequence the membrane, or at least the barrier portion, has a low electrical conductivity.

(ii) The respiratory chain is an alternating sequence of hydrogen carriers and electron carriers, arranged across the membranes in loops. Oxidation of a substrate results in the translocation of protons from one side of the membrane to the other: in any one loop, two protons pass across. The particular arrangement shown in Fig. 2 is taken from Skulachev (350); it includes one loop corresponding to the transhydrogenase reaction, and three for the oxidation of NADH.

For illustration consider the oxidation of NADH, corresponding to the first coupling site. Flavine is reduced at the inner surface but reacts at the outer surface with nonheme iron; protons are ejected while the electrons return to the inner surface. Here they are donated to coenzyme Q, together with a pair of

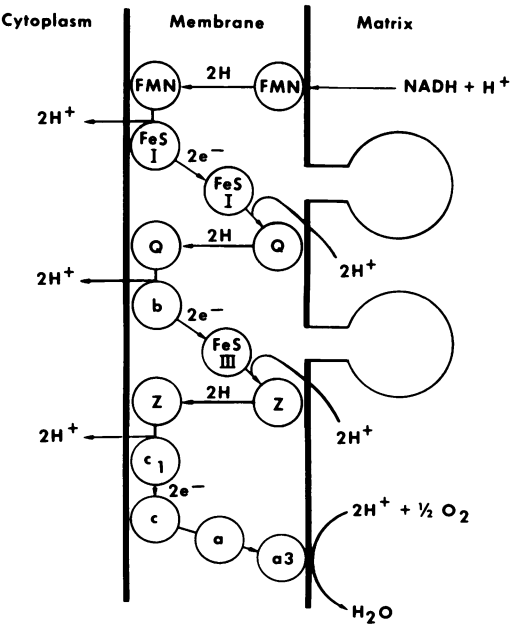

FIG. 2. *Pathway of proton and electron transfer during oxidation of NADH, according to the chemiosmotic hypothesis. After Mitchell (257), Greville (125), and Skulachev (350). Q, Coenzyme Q; Z, hypothetical hydrogen carrier; FeS, nonheme iron proteins; b, c, a, a_3, cytochromes.*

protons from the matrix fluid. In the second loop, the reduced coenzyme Q reacts with oxidized cytochrome *b* at the outer surface; protons are ejected, but the electrons return to the inner surface to enter the third loop. Overall, the passage of two reducing equivalents over each loop results in the appearance of two protons in the outer phase while two protons disappear from the inner phase. Oxidation of NADH-linked substrate will translocate a total of six protons.

Translocation of protons in one direction is equivalent, both in theory and in practice, to the movement of OH^- the other way. We can therefore state the postulate thus: oxidation of NADH results, not in the formation of water, but in the production of the elements of water, H^+ and OH^-, on opposite sides of an impermeable membrane.

The end result of substrate oxidation is the generation across the membrane of a gradient of *p*H and of electrical potential, with the matrix phase alkaline and electrically negative relative to the outer phase. Both gradients exert a force on the protons extruded by the respiratory chain, tending to pull them back across the membrane into the interior. This "proton-motive force" is the key element in energy coupling. It is essential to recognize that the proton-motive force is the sum of two components which are related but not identical: a chemical or osmotic component, due to the *p*H difference; and an electrical component due to the membrane potential. These are interconvertible, and it is convenient to express the proton-motive force in electrical units as the sum of the two components:

$$\Delta p = \Delta\Psi - Z\Delta p\mathrm{H}$$

(Δp is the proton-motive force in electrical units and can be taken as a measure of the electrochemical potential of protons. $\Delta\Psi$ is the electrical potential difference across the membrane. Z = 2.3 RT/F, where R, T, and F have their usual meanings, has a numerical value near 60 mv in the biological range. ΔpH is the *p*H difference between interior and exterior. If the inner phase were more alkaline than the exterior by one unit, and electrically negative to the extent of 180 mv, the total Δp would be −240 mv).

Obviously, a difference of *p*H or of electrical potential can be maintained only so long as the membrane forms a vesicle that is topologically closed; any defect or leak would dissipate the proton gradient.

(iii) The gradient of *p*H and of electrical potential generated by the respiratory chain reverses the direction of an ATPase so as to bring about net synthesis of ATP.

Mitochondrial membranes contain an ATPase which is inhibitable by oligomycin and DCCD; it is attached by a stalk to the inside of the cristae membrane and gives the inner surface its characteristic, knobbed appearance. The studies of Racker and his associates (summarized in 310, 311) leave little doubt that this enzyme catalyzes the terminal step in the biosynthesis of ATP. Now, ATPase is assayed by the hydrolysis of ATP, which normally proceeds virtually to completion; if the enzyme is to catalyze net ATP synthesis, something must drive the reaction in the opposite direction.

According to the chemiosmotic hypothesis, the ATPase catalyzes the obligatory and reversible translocation of protons: The reaction catalyzed bv the enzyme is to be represented, not as is usually done, (disregarding ionization):

$$\mathrm{ATP} + \mathrm{H_2O} \rightleftharpoons \mathrm{ADP} + \mathrm{Pi}$$

but by either of the following formulations:

$$\mathrm{ATP} + \mathrm{H_2O} + \mathrm{H^+_{in}} \rightleftharpoons \mathrm{ADP} + \mathrm{Pi} + \mathrm{H^+_{out}} \quad \text{(ATPase I)}$$

or

$$\mathrm{ATP} + \mathrm{H_2O} + 2\mathrm{H^+_{in}} \rightleftharpoons \mathrm{ADP} + \mathrm{Pi} + 2\mathrm{H^+_{out}} \quad \text{(ATPase II)}$$

If this is correct, the equilibrium constant of the ATPase should be written, not as it usually done:

$$\mathrm{Keq} = \frac{(\mathrm{ADP})\,(\mathrm{Pi})}{(\mathrm{ATP})}$$

but rather:

$$\mathrm{Keq} = \frac{(\mathrm{ADP})\,(\mathrm{Pi})}{(\mathrm{ATP})} \times \frac{(\mathrm{H^+})_{out}}{(\mathrm{H^+})_{in}} \quad \text{(for ATPase I)}$$

or

$$\mathrm{Keq} = \frac{(\mathrm{ADP})\,(\mathrm{Pi})}{(\mathrm{ATP})} \times \frac{(\mathrm{H^+})^2_{out}}{(\mathrm{H^+})^2_{in}} \quad \text{(for ATPase II)}$$

It is evident that the poise of the equilibrium would depend upon the proton activity on both sides of the membrane and thus upon the proton motive force. Therefore, a disequili-

brium of H^+ activity could, in principle, reverse the direction of the ATPase. Using certain values for the free energy of hydrolysis of ATP and for the steady-state concentrations of ATP, ADP, and Pi (all of these, incidentally, are subject to dispute), Mitchell arrives at the conclusion that an ATP/ADP ratio of 1 could be maintained by a ΔpH of 3.5 units, a $\Delta\Psi$ of -210 mv or any combination of lesser values adding up to the same total proton-motive force.

There is another, intuitively easier way to visualize the proposed function of the ATPase. We can say that the enzyme is so localized in the membrane that ATP, ADP, Pi, and H^+ have access to the active site only from the inside, OH^- only from the outside, and water as such no access at all. Synthesis of ATP requires "extraction" of water from ADP and Pi, but it is in fact the *elements* of water that are extracted: H^+ would be pulled inward, and OH^- outward, by the gradient of *p*H and electrical potential established by the respiratory chain. By either formulation, synthesis of ATP is associated with movement of protons inwards, completing the circuit of a proton current which can be regarded as the driving force for ATP synthesis (Fig. 3).

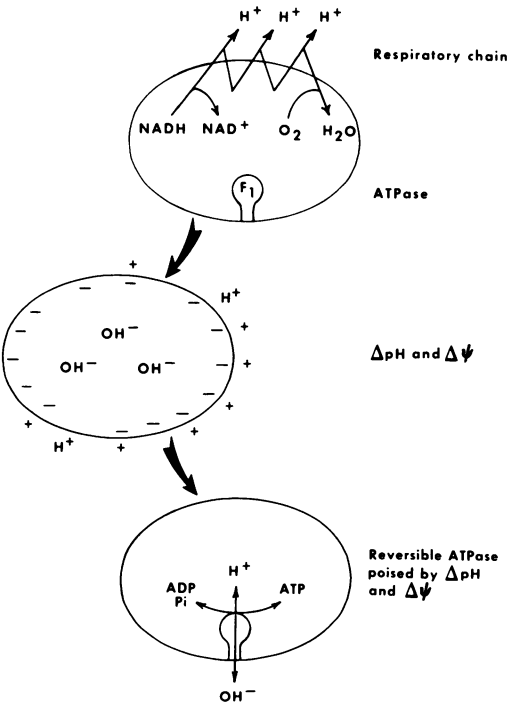

FIG. 3. *Chemiosmotic hypothesis in principle: extrusion of protons by the respiratory chain, generation of ΔpH and $\Delta\psi$, and the poising of ATPase by the proton-motive force.*

It is now necessary to specify in some chemical detail just how the hydrolysis of ATP is reversibly coupled to the translocation of protons across the membrane. This is not presently possible, nor is it known with certainty for any system whether ATP hydrolysis is accompanied by the translocation of one or of two protons. Mitchell's original proposal (257) invoked an anhydride structure, X ~ I, and the translocation of ionizable groups belonging to a (protein?) component of the system. These have been critically discussed by Greville (125), Racker (310), and Skulachev (350). A more recent formulation (266) dispenses with the anhydride in favor of closely specified pathways for the conduction of protons through the system. Detailed consideration of what must still be regarded as a purely speculative mechanism seems out of place here: In Greville's words (125), "Until more is known about the structures and enzymatic mechanisms of the components, ... the postulated proton-translocating ATPase system with its unidentified groups XH and IOH will remain the least objective part of the Mitchell hypothesis."

According to the chemiosmotic hypothesis, the proton-motive force is the common factor responsible not only for the synthesis of ATP but also for the other energy-linked functions of mitochondria. In later sections we shall consider in detail the role of *p*H gradients and membrane potentials in the transport of cations, anions, and substrates.

It may be useful at this point to bring out the essential differences between the chemical and the chemiosmotic interpretations of mitochondrial function. This is not primarily a matter of intermediates: unidentified and elusive intermediates have been invoked by both. There remain, however, two fundamental differences. The chemical hypothesis postulates chemical linkage between the oxidation chain and the ATPase; according to Mitchell, these processes are coupled only via the proton-motive force and do not share a chemical intermediate. For this reason, the demonstration that a gradient of *p*H or of electrical potential (or both) does in fact exist is a touchstone of the chemiosmotic hypothesis (without necessarily being incompatible with other explanations). At a more generalized level, the Mitchell theory postulates vectorial metabolism both within and across the membrane and requires that phosphorylation be dependent

upon the topological integrity of the system.

Point and Counterpoint

Efforts by the protagonists to rigorously disprove one or another of the adversary positions outlined above have generated hundreds of research papers during the past decade. An observer of pacific inclinations may derive some satisfaction from the gradual melding of views which appeared utterly irreconcilable at first. Proposals have also been formulated which embody elements of all three hypotheses. Williams, for example (393, 394), stresses the generation of protons as the primary event, but within the membrane rather than across it; in the hydrophobic lipid phase, bare protons would serve as dehydrating agents to drive the synthesis of ATP. The historical fact is, however, that it is the chemiosmotic and chemical coupling hypotheses that led to predictions verifiable by experiment, and it is principally in terms of these hypotheses that we shall examine the experimental observations.

The following survey, of necessity selective and incomplete, deals chiefly with controversies that have arisen (or will arise shortly) in the context of bacterial membranes. Many issues, including those generated by measurements of redox potentials and of other thermodynamic parameters, have been omitted altogether since no equivalent data from microorganisms are presently available. Thus I do not purport to judge between the rival theories: Racker (310), Skulachev (350), and Slater (352) have complied "scoreboards" which reveal the degree to which this matter is still *sub judice*. The intention is rather to outline the basis for the belief that principles embodied in the chemiosmotic hypothesis offer valid insights into membrane function even if the present formulation of the hypothesis should need to be revised.

The present section is chiefly concerned with the nature of the energized state in relation to oxidative phosphorylation. Membrane transport will be considered in a separate section, followed by a summary.

Permeability of the mitochondrial membrane to protons. Ever since the recognition that the slow oxidation of exogenous NADH by mitochondria is due to a permeability barrier, it has been generally admitted that the mitochondrial membrane excludes a variety of small molecules. Translocation of molecules which participate in mitochondrial metabolism, such as orthophosphate (Pi), ADP, ATP, and various substrate anions is known to be mediated by functionally specialized transport systems which will be discussed below. The chemiosmotic hypothesis requires, however, that the membrane be also impermeable to H^+ and OH^-: were it permeable to these ubiquitous ions, gradients of *p*H and of electrical potential could not be sustained. The effective proton conductance, i.e., the sum of the permeability to H^+ and OH^-, was measured by Mitchell and Moyle (267) by following the rate of hydrogen ion titration across the mitochondrial membrane and was found to be very low indeed, 0.45 μmho/cm^2 or 0.11 μg of H^+ per sec per *p*H unit per g of mitochondrial protein.

For a number of years, Chance and his associates denied that the mitochondrial membrane is impermeable to protons, for both theoretical and experimental reasons (59, 65, 66). In a more recent paper (272), however, the essential ion impermeability of the membrane is taken for granted, and this may now be regarded as generally accepted. Artificial phospholipid bilayer membranes are also exceedingly impermeable to protons, which is most likely a general property of lipid membranes.

Vectorial organization of respiratory catalysts. It is an essential postulate of the chemiosmotic hypothesis that membrane catalysts, including both the oxidation chain and the ATPase, are organized across the membrane and catalyze vectorial metabolism. Some of the earliest evidence bearing on this point came from the recognition that the membrane as a whole has a definite *polarity*.

When mitochondria are negatively stained and examined by electron microscopy, the matrix side is seen to be lined with spherical particles attached to the membrane proper by a stalk. These were initially referred to as elementary particles and believed to contain the electron transport chain. More recent work from Racker's laboratory, however, makes it virtually certain that the stalked particles represent only the coupling device; the spherical particles are identical with coupling factor CF_1 and possess ATPase activity (310, 311).

Disruption of the mitochondria yields two kinds of submitochondrial particles, both of which still carry out oxidative phosphorylation. Particles prepared with the use of digitonin have the same polarity as do the original mitochondria, but those produced by ultrasonic disruption appear to be inside-out. This is the simplest explanation of the finding that in sonic particles the direction of many mito-

chondrial functions is the reverse of that seen in intact organelles. Among these are the localization of the stalked particles, which now face the medium; the direction of proton translocation, which is inward in the particles (155, 257, 260, 262); the polarity of ion movements, which indicates that the particles generate an electrically positive interior whereas that of the parent mitochondria is negative (126, 272, 349, 350); and the accessibility of cytochromes, ATPase, and other enzymes to substrates, inhibitors, antibiotics, or solvent extraction.

The latter characteristic has been exploited in several laboratories, but especially by Racker and his associates, to dissociate the components of the oxidation chain and coupling device and to reassemble them in their proper order and topological orientation (reviewed in 310, 311, 350, 377). This work leaves no doubt that the oxidation chain is, in fact, arranged across the membrane. To accomodate the information presently available, a minimum of one loop is required, which places the dehydrogenases and cytochrome oxidase near the matrix surface, cytochrome *c* near the surface facing the cytoplasm. The actual organization may well prove to be more complex as more data accumulate.

One very striking conclusion to emerge is that some of the components play a structural as well as a catalytic role and that reconstitution of oxidative phosphorylation is invariably associated with recovery of a topologically closed vesicle (310, 311). In summary, there is mounting evidence that proper topological orientation of all the components is, in fact, essential to phosphorylation though not necessarily to oxidation. This, like the association of phosphorylation with a vesicular structure, confirms general insights of the chemiosmotic hypothesis. However, the actual sequence of electron and hydrogen carriers and their particular orientation remain the subject of much dispute.

Proton extrusion and the generation of a membrane potential. According to the chemiosmotic hypothesis, the oxidation chain is so arranged as to catalyze the extrusion of protons, thereby generating a difference of *p*H and of electrical potential which in turn poises the equilibrium of the ATPase. Since the proton-motive force is said to be the only link between respiration and phosphorylation, demonstration of the existence of such gradients becomes crucial. This is especially so for mitochondria respiring in the absence of ADP, since under these conditions the electrical potential ought to be maximal.

There is no doubt that respiring mitochondria do eject protons, by a very rapid process (155, 263, 268, 304). Mitchell and Moyle reported that, upon admission of a pulse of oxygen to an anaerobic suspension of mitochondria, 6 protons were extruded per mole of an NADH-linked substrate, 4 protons per mole of succinate (262, 263, 268); the extrusion of protons, if not always the same stoichiometry, has been confirmed in other laboratories (e.g., 155).

The catch is that proton extrusion by mitochondria proved to be linked to concomitant accumulation of Ca^{2+}. It now appears clear that Ca^{2+} leaks out of the organelles during anaerobiosis and is reabsorbed when oxygen is admitted (reviews: 125, 235, 377). It is therefore uncertain which is primary, the extrusion of protons or the transport of Ca^{2+}; we shall return to this problem below.

Tupper and Tedeschi (370–372) made a heroic attempt to resolve the issue by direct determination of the electrical potential across the mitochondrial membrane. They impaled the giant mitochondria of *Drosophila* salivary glands upon microelectrodes and recorded a small potential, interior *positive* and independent of metabolism. These results flatly contradicted the chemiosmotic hypothesis and were, in fact, predicted by one version of the chemical hypothesis (143). However, considering the enormous size of the microelectrode relative to its target and the surprisingly low value reported for the electrical resistance of the membrane, the possibility of mechanical damage is all too real.

It seems to this reviewer that, despite the indirect approach, measurements of the electrical potential based on ion movements carry more conviction. If the respiratory chain generates an electrical potential, interior negative, mitochondria should tend to accumulate cations and expel anions; the reverse should apply to sonic particles which are inside-out. It is important to study ions which, unlike Ca^{2+}, are not normally transported. The first application of this principle was made by Mitchell and Moyle (260, 263, 270). Mitochondria do not ordinarily take up K^+ but can be induced to do so by addition of the antibiotic, valinomycin. The explanation generally given is that valinomycin forms a lipid-soluble complex with K^+ and thus renders the membrane, to all practical purposes, freely permeable to K^+ (for reviews see 132, 150, 277, 307, 308). Therefore, K^+ added to respiring mitochondria should distribute itself in accordance with the electro-

chemical potential. A negative potential would lead to K^+ accumulation as required by the Nernst equation:

$$\Delta\psi = \frac{RT}{F} \ln \frac{[K^+]_o}{[K^+]_i}$$

(In this equation, $\Delta\psi$ is the membrane potential, $[K^+]_o$ and $[K^+]_i$ refer to the potassium concentration on the outside and inside, respectively, and R, T, and F have their usual meanings; activity coefficients are neglected.) In fact, valinomycin-treated mitochondria accumulate K^+ to an extent consistent with a potential of some -250 mv, interior negative (263, 270, 325); it is only fair to mention that Pressman and his associates gave the induction of K^+ accumulation by valinomycin quite another interpretation (307, 308).

The most convincing series of experiments is due to Skulachev, Liberman, and their associates (22, 126, 238, 241, 349, 350) who studied the translocation of synthetic, lipid-soluble cations and anions. The structures of some of these are given in Fig. 4. Briefly, intact respiring mitochondria were shown to accumulate the cations and expel the anions, as would be predicted for an electrically negative interior. Conversely, sonic submitochondrial particles accumulate the anions but expel the cations. The results can hardly be attributed to active transport of these nonphysiological ions; generation of a membrane potential by mitochondrial membranes thus appears to have been demonstrated (238, 349, 350). The potential could arise by a proton-translocating oxidation chain, as proposed by the chemiosmotic hypothesis, or by some other process; the alternatives are discussed at length in Skulachev's valuable review (350).

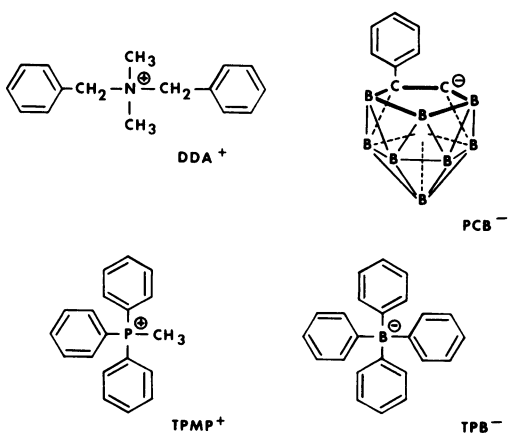

FIG. 4. *Lipid-soluble synthetic ions. DDA+, dibenzyldimethyl ammonium; TPMP+, triphenylmethylphosphonium; PCB−, phenyldicarbaundecaborane; TPB− tetraphenyl boron.*

The coupling device: ATPase and ion translocation. The coupling device, which mediates the energy-transfer reactions that culminate in the synthesis of ATP, has proven to be a complex and sophisticated system. The ATPase proper, or F_1 particle, is a large protein (probably a hexamer) composed of subunits of several kinds (62, 115, 343, 375). At least four additional proteins, and at least one phospholipid, are required to reconstitute normal coupling function in submitochondrial particles: one of the proteins inhibits, or masks, the ATP hydrolase activity. Inhibitors of the energy-transfer functions have proven to be invaluable for analysis of the coupling device: oligomycin, rutamycin, and DCCD bind not to the ATPase itself but to other components of the system. Inhibition of the ATPase function is indirect and may reflect transmitted effects on conformation. Other inhibitors, including aurovertin and Dio 9, appear to interact with the F_1 particle itself (60, 61, 218; reviews: 132, 151, 310).

It has long been apparent from studies with inhibitors that the coupling device is an element quite separate from the respiratory chain. Indeed, oxidation chain and coupling device can be physically dissociated: Groot et al. (127) found that promitochondria of anaerobic yeast (which lack cytochromes as well as ubiquinone) still carry out various energy transfer reactions involving ATPase. Conversely, Kagawa and Racker (193) recombined ATPase, phospholipids, and an amorphous fraction devoid of respiratory activity to restore vesicles which exhibited ATP-dependent, energy-linked functions.

How this multienzyme system functions in the synthesis of ATP is unknown. Various partial and exchange reactions, such as the ADP/ATP exchange, are most easily understood in terms of a mechanism that invokes discrete steps in ATP synthesis, as does the chemical coupling hypothesis (42, 310). The case is strengthened by the detection (82) of a phosphorylated intermediate of the X~P type, which could be the elusive phosphoryl donor to ADP. Fisher et al. (111) have reported a solubilized ATP synthetase complex which catalyzes various exchange reactions in a manner sensitive to uncouplers and oligomycin; they suggested that this preparation gives rise to at least some of the intermediates of oxidative phosphorylation even though it is apparently

devoid of membranes.

According to the chemiosmotic hypothesis, the ATPase reversibly translocates protons across the membrane. Hydrolysis of ATP should elicit electrogenic extrusion of protons from mitochondria, and conversely, imposition of an appropriate gradient of *p*H or potential should bring about net ATP synthesis. Both of these predictions have been confirmed.

(i) The demonstration that a *p*H gradient imposed on chloroplasts in the dark resulted in ATP synthesis (177) was an early and impressive vindication of the chemiosmotic hypothesis. Similar, but less dramatic, findings were made with mitochondria (315). ATP synthesis could also be supported by a K^+ gradient: Efflux of K^+ from mitochondria, induced by valinomycin, results in ATP synthesis with a stoichiometry so high as to be difficult to explain by the current version of the chemiosmotic hypothesis (73, 322).

(ii) Addition of ATP to an anaerobic suspension of mitochondria led to ejection of protons, whereas sonic particles absorbed protons under these conditions. The stoichiometry is uncertain (257, 262, 269). This is accompanied by uptake of a cation, and little proton extrusion is seen unless a cation is present—Ca^{2+}, or valinomycin plus K^+. Therefore, it is of crucial importance to the interpretation of these experiments that ATP hydrolysis supports uptake of lipid-soluble cations by mitochondria and of anions by the particles (22, 126, 238). It follows that ATP hydrolysis generates the predicted electrical potential, presumably by electrogenic movements of protons.

To the extent that the results were predictable, in principle if not in detail, from the chemiosmotic hypothesis, they must count in its favor. However, they are generally compatible with Fig. 1, regardless of the nature of "~" so long as we take respiration, ion gradients, and ATP to be reversibly interconnected. Therefore, dramatic and important as these experiments are, they do not permit an unambiguous choice between the mechanisms under consideration.

Uncoupling and proton conduction. Mitochondria are easily damaged by rough handling, detergents, and other treatments, all of which dissociate respiration from phosphorylation to a greater or lesser degree. Interest centers, however, upon the chemical uncouplers which act at very low concentrations; 2,4 dinitrophenol was the first of these to be discovered and remains the most familiar. Typically, uncouplers block phosphorylation but stimulate respiration; high concentrations of the uncoupler may accelerate the dissipation of X ~ I, for instance by promoting the hydrolysis of an energy-rich intermediate (351).

A major clue to the mode of action of uncouplers was supplied by the recognition that many uncouplers are lipid-soluble acids whose *p*K is such that at physiological *p*H values both the protonated form and the anion will exist in substantial proportions (for structures, see Fig. 5). Mitchell (255–257, 260, 261, 264) proposed that uncouplers dissolve in the membrane and act as circulating carriers conducting protons across the barrier. Diffusion of protons would dissipate the proton gradient on which oxidative phosphorylation depends, and relieve the restriction of respiration.

During the past decade, much experimental evidence has accumulated to support the thesis that many uncouplers are proton conductors. By use of their titration technique, Mitchell and Moyle (267) demonstrated that carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and other uncouplers enhance the rate of proton diffusion across the mitochondrial cristae membrane by several orders of magnitude. The rate of proton diffusion in the presence of uncouplers is sufficient to account for the uncoupling in terms of the chemiosmotic hypothesis (155, 166, 241, 267, 268, 360). Compelling evidence for proton conduction by uncouplers came from studies with artificial lipid bilayers. Uncouplers increase the electrical conductivity and induce an electrical potential across a membrane separating two compartments which differ in *p*H (166, 239, 240). The

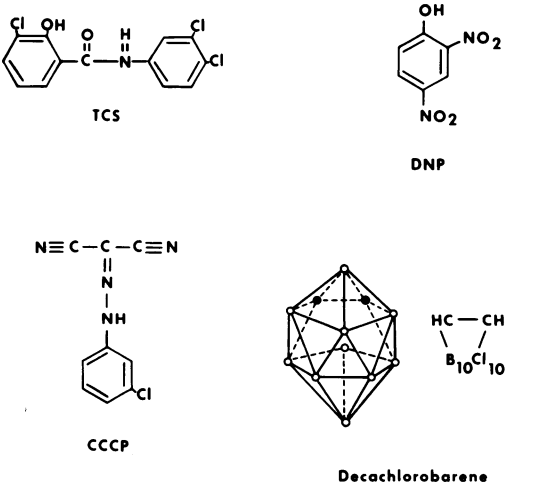

FIG. 5. *Proton-conducting uncouplers. TCS, tetrachlorosalicylanilide; CCCP. carbonylcyanide m-chlorophenylhydrazone; DNP, dinitrophenol; decachlorobarene. Symbols:* ●, *CH;* O, *BCl.*

results suggest that uncouplers enhance the diffusion of H^+ (or OH^-) specifically and have little effect on the diffusion of other ions. The precise mechanism of proton conduction is still controversial. It has been suggested that the species which carries the current is the dimer of the undissociated and the dissociated species, $HA.A^-$ (110). Most investigators, however, favor Mitchell's original proposal that the uncoupler travels one way as the protonated acid and the other way as the anion. The structures of the anions (Fig. 5) tend to delocalize the charge and enhance solubility of the anion in the lipid phase (165, 231, 239, 257, 261, 264).

It should be pointed out that the proton conductors shown in Fig. 5 carry out an electrogenic transport of protons. This stands in contrast to antibiotics, such as nigericin and monensin, which catalyze antiport of H^+ for K^+ or Na^+ (reviews: 132. 150, 277, 307, 308). The latter antibiotics do not enhance the electrical conductivity of a membrane, nor are they uncouplers—presumably because they do not dissipate the electrical potential. On the other hand, valinomycin, which conducts K^+, *is* an uncoupler under certain conditions, because influx of K^+ dissipates the potential (261, 325).

Despite the general acceptance of the phenomenon of proton conduction, it is by no means universally accepted that uncoupling is the consequence of diffusion of protons *across* the membrane. It will be recalled that the chemical theory proposes that these two processes are linked by energy-rich intermediates which normally exist in a lipid, hydrophobic environment. Proton conductors may well catalyze hydrolysis of such compounds by allowing access of protons to the active site (166, 361). Wilson and his associates (367, 396) have examined in detail the *p*H profiles of uncoupling and proton conduction. The two are not the same, and the authors concluded that uncoupling is not the result of proton conduction but rather is due to general acid or base catalysis of a hydrolytic reaction taking place in the membrane matrix (but see also 165). In this connection, it should be recalled that many uncouplers bind to proteins of the mitochondrial membrane (383), and this could also play a role in uncoupling. In summary, while there is no question that many uncouplers do conduct protons across lipid membranes, it seems impossible at this time to say whether uncoupling results from passage of protons *across* or *into* the coupling membrane.

Fluorescent molecules as probes of the energized state. The fluorescence of certain molecules, such as ANS^- (1-anilino-8-naphthalene sulfonate), is greatly enhanced if the substance is localized in a hydrophobic environment. This property rendered ANS^-, which readily binds to proteins, useful in detecting changes in protein conformation. The recent application of fluorescent dyes of this kind to mitochondria provides a new tool with which to probe the nature of energy coupling.

Briefly, binding of ANS^- to submitochondrial particles results in some enhancement of fluorescence. Addition of substrate or of ATP produces further enhancement, which can be blocked by uncouplers. The fluorescence response lags behind the change in oxidation state of the respiratory carriers. From these and other experiments, it was inferred that ANS^- responds to the energized state of the mitochondrion, in much the same way as do other energy-linked functions. The enhancement of fluorescence was traced partly to an increased affinity for the dye, resulting in additional binding, partly to increased quantum yield of the fluorescence of bound dye. Clearly, ANS^- fluorescence reports a change in the state of the mitochondrial membrane—perhaps a change in the conformation of the membrane, or the advent of a more hydrophobic state due to extrusion of water. The response of ANS^- is too slow to reflect the primary energy-conserving event (19, 48, 64, 83).

Of great importance to the interpretation of ANS^- fluorescence is the recognition that in this, as in so many other respects, the effects seen with whole mitochondria are opposite in polarity to those of particles. The fluorescence of ANS^- associated with intact mitochondria was *decreased* when the organelles were energized. Moreover, the fluorescent cationic dye Auramin-O was found to respond in a manner opposite to that of the anionic ANS^-: fluorescence and binding were decreased in particles and increased in intact mitochondria (17). Thus, the fluorescence response is a function of the sidedness of the membrane and of the distribution of electrical charges either within or across the membrane. Indeed, ANS^- fluorescence responded to the artificial induction of a membrane potential in the sense that a potential, interior negative led to a decrease in ANS^- fluorescence (20, 179).

These very recent results led to two possible interpretations, both of which emphasize the amount of ANS^- associated with the (hydrophobic) mitochondrial membrane. Azzi et al. (20) suggested that when mitochondria are

energized the inner (matrix) side becomes more positive and the outer side more negative. In particles, it is the matrix side which faces the medium, resulting in increased binding of ANS^-, an anion, and enhanced fluorescence. Quite possibly the charge redistribution involves specific regions of the membrane related to the coupling sites, rather than the bulk of the membrane phase. An alternative interpretation was favored by Jasaitis et al. (179): they argued that the amount of ANS^- bound to the membrane is a function of the concentration of dye in the mitochondrial matrix, and this in turn reflects the electrical potential across the membrane. ANS^- would behave like the lipid-soluble anions and cations discussed earlier: Submitochondrial particles develop an electrically positive interior; as ANS^- migrates into the particles in response to the potential, a larger fraction becomes associated with the membrane and enhanced fluorescence ensues.

Metabolite Transport by Mitochondria

To one accustomed to the microbiological transport literature, that on mitochondria conveys quite another flavor since it gives pride of place to the relationship of metabolite translocation to oxidation and energy transduction. The mitochondrial literature is thus worth examining precisely for the lessons that its unique focus may hold for those concerned with transport in cells.

The cristae membrane appears to be freely permeable to some major metabolites, including water, oxygen, CO_2, and pyruvic acid, but is otherwise a barrier to diffusion. A variety of transport systems breach this barrier, both primary and secondary. The primary systems for proton translocation were discussed in the preceding section. In addition, there is evidence that the uptake of glutamate, aspartate, and fatty acids occurs by group translocation. Among secondary systems, the best studied is the porter for adenine nucleotides, but since it appears to have no equivalent in microorganisms it will not be considered here. This section will focus instead on the accumulation of cations, anions, and substrates and its relationship to the energized state of the mitochondrial membrane. The burgeoning literature on metabolite transport in mitochondria has been reviewed by Lehninger et al. (235), Pressman (308), Chappell (67), Klingenberg (215), and by Van Dam and Meyer (377).

Accumulation of calcium. Most animal mitochondria rapidly accumulate divalent cations by an energy-linked process. Only that of Ca^{2+}, which has been studied most thoroughly, will be considered here.

There is overwhelming evidence that Ca^{2+} uptake is mediated by a specific carrier of high affinity, which is under genetic control. Ca^{2+} binding by liver mitochondria has an apparent dissociation constant of about 10^{-6} M and is specifically inhibited by lanthanum and by praesodymium (232, 234, 250, 376). Mitochondria of yeast (55) and of the blowfly (56), which lack the high-affinity binding sites, also lack the characteristic, rapid Ca^{2+} accumulation. Recently, Lehninger (233) reported the release, after osmotic shock, of a protein which binds Ca^{2+} in a manner identical with that of the whole mitochondria. This protein, reminiscent of the binding proteins of bacterial cells but of much greater molecular weight, may mediate the initial step in Ca^{2+} uptake.

There is a voluminous literature on the relationship of Ca^{2+} uptake to oxidation, which can only be summarized here (see also: 232, 235, 308). Briefly, addition of a limiting amount of Ca^{2+} to respiring mitochondria elicits a burst of respiration, which ceases when all the Ca^{2+} has been taken up. Concomitantly, protons are ejected and the internal *p*H of the mitochondria rises by a unit or more (7, 121). The precise stoichiometry depends on conditions, but a ratio of 2 Ca^{2+} per electron pair passing each coupling site is typical. These experiments are conducted in absence of a permeant anion; the Ca^{2+} taken up, maximally about 100 μmoles per g of mitochondrial protein, remains associated with the mitochondrial membrane.

Much larger amounts of Ca^{2+} can be accumulated in presence of an anion which traverses the barrier—phosphate or acetate, for example. Alkalinization of the matrix and proton ejection are suppressed, and Ca^{2+} accumulates in the matrix in form of a salt: when acetate is the anion, the mitochondria swell, even to the point of lysis. Phosphate, however, allows the precipitation of internal calcium phosphate, a surprisingly complex process which is still imperfectly understood.

Ca^{2+} uptake is supported by respiration and blocked both by inhibitors of the chain and by proton-conducting uncouplers; significantly, Ca^{2+} is discharged by these inhibitors. Oligomycin and DCCD do not inhibit uptake. However, with the respiratory chain blocked, Ca^{2+} uptake can be energized by ATP: for each molecule of ATP hydrolyzed, about 2 mole-

cules of Ca^{2+} and 1 molecule of Pi are absorbed. The ATP-supported uptake, unlike that supported by respiration, is blocked by oligomycin and DCCD. These observations and earlier ones on the uptake of Mg^{2+} by heart mitochondria (see reference 235 for review) are of crucial importance to the mapping of energy transfer pathways: they point to an energized state, or intermediate, *other* than ATP, as the driving force for Ca^{2+} uptake (Fig. 1).

When we inquire into the nature of the energizing event, we quickly find ourselves enmeshed in arguments related to the chemical and chemiosmotic coupling theories. No fewer than three schemes, summarized in Fig. 6, were formulated to account for the relationship of proton efflux to cation uptake.

(i) According to Mitchell (257, 260, 262, 263), uptake of Ca^{2+} is electrophoretic, in response to the membrane potential generated by the respiratory chain. Indeed, since the electrogenic extrusion of even a very few protons generates a large potential, significant *net* H^+ ejection is to be expected only in presence of a cation which can enter the mitochondrion and thus compensate for the electrical displacement.

(ii) Chance and his associates (65, 66, 308) championed scheme b, in which the hypothetical energy-rich intermediate, X ~ I, pumps Ca^{2+} inward while protons are expelled due to the membrane potential, interior *positive*. This scheme assumes a proton-permeable mitochondrial membrane.

(iii) Finally, scheme c introduces the concept of a proton pump, actuated indirectly by the oxidation chain via an energy-rich X ~ I intermediate. As written here, this pump extrudes protons to generate an electrical potential, interior negative, which in turn drives the electrophoretic uptake of Ca^{2+} (68, 69, 238, 350). Alternatively, one might envisage obligatory linkage between the movements of protons and of Ca^{2+}—i.e., an energized exchange of Ca^{2+} for protons.

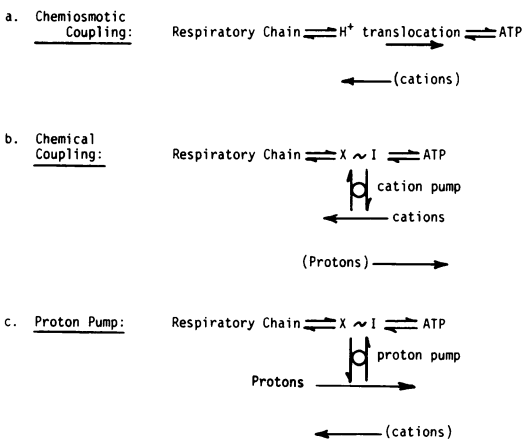

FIG. 6. *Possible interrelationships of cation and proton translocations.*

With the realization that the mitochondrial membrane is not readily permeable to protons and that the interior is electrically negative, scheme b has been eliminated. It is more plausible that a chemically driven proton pump may be linked to the Ca^{2+} carrier (Fig. 6c) in such a way that the exchange of H^+ for Ca^{2+} is, overall, electrically neutral. But there seems to be a growing measure of agreement that proton ejection is the primary event. The protons may be translocated by a vectorial respiratory chain, or by a proton pump energized by an X ~ I intermediate. By either scheme, we can account for the characteristics of Ca^{2+} uptake on the assumption of electrophoretic Ca^{2+} uniport in response to the electrical potential (232).

Accumulation of potassium. Unlike the rapid and extensive accumulation of K^+ so characteristic of bacteria, uptake of K^+ by mitochondria is sluggish—limited by the low permeability of the mitochondrial membrane to K^+. Addition of ion-conducting antibiotics of the valinomycin type, however, induces massive and rapid uptake of K^+ (68, 306).

The extensive literature concerning the nature and mode of action of ionophores has been repeatedly reviewed in recent years (132, 150, 277, 307, 308), so that a quick sketch will suffice here. Briefly, in addition to the proton conductors discussed above, we recognize two classes of alkali-metal ionophores (Table 1).

(i) *Valinomycin* is the type species of a K^+-specific uniporter. The molecule is a cyclic depsipeptide which forms a clathrate such that K^+ is encaged in the center of a shell whose exterior is hydrophobic. The complex is consequently lipid-soluble and functions as a circulating carrier for K^+. It must be noted that the complex as a whole bears a positive charge, so that net K^+ movement is electrogenic: it both generates, and responds to, an electrical potential. In other words, valinomycin moves K^+ in accord with the electrochemical potential.

Valinomycin is exceedingly specific for K^+. Enniatins and the macrotetralide "nactins" are less selective but, like valinomycin, they act as circulating carriers by formation of lipid-soluble clathrates. Gramicidin, which is quite promiscuous, apparently conducts cat-

ions by forming a cation-selective pore.

(ii) *Nigericin* is the prototype of a second class of ionophores, all of which are monocarboxylic acids. They were originally recognized by virtue of their capacity to reverse the action of valinomycin—that is, to discharge K^+ from mitochondria. Their mode of action is now known to depend again on formation of a lipid-soluble clathrate. However, it is the anion of nigericin that complexes K^+ to give an electrically neutral complex; the protonated nigericin does not bind K^+. Consequently, the antibiotic tends to carry out exchange of K^+ for H^+, or K^+/H^+ antiport. The related antibiotic monensin catalyzes Na^+/H^+ antiport.

The characteristics of the K^+ uptake induced by valinomycin are qualitatively similar to those of Ca^{2+}. Translocation, which occurs against a large concentration gradient, is energy linked and can be supported either by respiration or by ATP. Uncouplers prevent K^+ accumulation and discharge K^+ already accumulated. Uptake of K^+ is electrically compensated by ejection of protons or by concurrent accumulation of anions (for summaries of the extensive studies see 69, 70, 125, 274, 307, 308). And the quest for the mechanism of energy input leads us back to Fig. 6.

The simplest interpretation now available is based on the chemiosmotic hypothesis (68, 69, 152, 176, 260, 270, 272): respiration and ATP hydrolysis both generate an electrical potential, interior negative. Accumulation of K^+ in presence of valinomycin is due to the well established capacity of the antibiotic to conduct K^+ across lipid membranes. Nigericin mediates K^+ efflux by exchange for protons from the medium; proton conductors dissipate the electrical potential by allowing protons to flow in and thus elicit the same result.

Interpretations of K^+ accumulation which rely upon chemical coupling have also been

TABLE 1. *A potpourri of inhibitors, antibiotics, and reagents which affect membrane processes*

Metabolic region	Inhibitor	Mode of action
Respiratory chain	Cyanide	Inhibits cytochrome oxidase
	Azide	Inhibits cytochrome oxidase, often ATPase as well; conducts protons
	Rotenone; piericidin	Specific inhibitors of first coupling site, probably on oxygen side of coenzyme Q
	Antimycin; HOQNO	Specific inhibitors of second coupling site, between cytochromes *b* and *c*
ATPase	Oligomycin, rutamycin	Typically inhibits mitochondrial, but not bacterial ATPase; site of action, the "oligomycin-sensitivity-conferring-protein"
	DCCD	Inhibits both mitochondrial and bacterial ATPases; reacts covalently with a protein component of the membrane
	Dio 9	Inhibits mitochondrial and bacterial ATPases; apparently binds to the ATPase itself
Ionophores		
H^+	Dinitrophenol CCCP, FCCP, TCS	Conduct H^+ very specifically across artificial and biological membranes; uncouple oxidative phosphorylation; H^+ movement is electrogenic
K^+	Valinomycin, monactin	Conduct K^+ very specifically across artificial and biological membranes. K^+ movement is electrogenic; do not always uncouple oxidative phosphorylation
K^+, Na^+ H^+	Gramicidin	Relatively nonspecific for monovalent cations; uncouples oxidative phosphorylation
K^+/H^+	Nigericin	Mediates electrically neutral exchange of K^+ for H^+; not usually an uncoupler
Na^+/H^+	Monensin	Mediates electrically neutral exchange of Na^+ for H^+; not usually an uncoupler
Lipid-soluble ions	DDA^+, $TPMP^+$	Lipid-soluble cations (Fig. 4); accumulated by, and uncouple, intact mitochondria
	TPB^-, PCB^-	Lipid-soluble anions (Fig. 4); accumulated by, and uncouple, submitochondrial particles

formulated. Massari and Azzone (246, 247, 322) propose a chemically driven proton pump which, however, carries out an obligatorily neutral exchange of H^+ for another cation (Ca^{2+}, perhaps). Valinomycin renders this carrier accessible to K^+ but would not be acting across the membrane, and a membrane potential is not invoked. The model derives from a somewhat earlier one, developed in detail by Pressman (308). Pressman postulated a carrier which forms a positively charged complex with cations—analogous to the valinomycin-K^+ complex. This carrier, whose function may be to drive uptake of *anions* against the electrochemical potential (i.e., an "anion pump") is thought to be driven by an X ~ I, energy-rich intermediate. Ordinarily, K^+ is denied access to the pump which is buried in the membrane lipid, but valinomycin lets K^+ pass to the active site. Carriers of this type can in principle account for the whole range of cation and anion translocations, but the mode of energy coupling must be specified more precisely than has yet been done.

Transport of phosphate and substrate anions. The recognition of specific carriers for anions resulted initially from application of osmotic swelling techniques. Mitochondria whose respiration is blocked are osmotically stable in 0.15 M KCl, because the membrane is impermeable to both ions, but they swell in ammonium phosphate or ammonium acetate. From studies on the effect of ionophorous agents, it was concluded that the entry of phosphate is an electrically neutral process which can be formulated either as Pi^-/OH^- antiport or else as Pi^-/H^+ symport (67, 69, 70, 271). Existence of a porter specific for phosphate and arsenate was confirmed by the discovery that translocation of these metabolites is specifically inhibited by certain mercurials (114, 373, 374).

Most of the experiments on phosphate uptake by mitochondria were done in the presence of inhibitors both of respiration and of ATP utilization. Despite the lack of any energy source, such mitochondria accumulate Pi against a substantial concentration gradient. The accumulation is strongly dependent on the external *p*H (for example, $[Pi]_i/[Pi]_o$ is 30 at *p*H 6 but only 10 at *p*H 8 (298). Accumulation is inhibited by proton conductors and by nigericin, but not by cation conductors. These and other results suggest that the accumulation of phosphate by nonenergized mitochondria depends entirely on the establishment of a *p*H gradient across the membrane. Measurements of proton movements and of the intramitochondrial *p*H have confirmed that almost two protons accompany each phosphate ion: uptake of phosphate results in alkalinization of the medium; efflux of phosphate results in alkalinization of the mitochondrial interior (Fig. 7a). Whether we regard the process as Pi-H^+ symport or as Pi^-/OH^- antiport, the internal phosphate level is a function of the *p*H gradient across the membrane, according to the relationship:

$$\log \frac{[An^{n-}]_i}{[An^{n-}]_o} = n\Delta pH$$

(where n is the valence of the anion and $\Delta pH = pH_i - pH_o$; see references; 162, 215, 248, 298, 300).

Uptake of phosphate, and indeed of anions generally, is enhanced if the mitochondria are permitted to respire, and especially so when a cation is provided: Ca^{2+}, say, or K^+ together with valinomycin. How are we to envisage the coupling of respiration to transport of phosphate? Harris and Pressman (143) suggested that cation uptake is the primary event and the anions follow passively. This scheme (Fig. 6b) predicts the generation of a positive potential and is at variance with most of the data currently available. Alternatively, active transport of phosphate has been attributed to primary anion pumps (see, for example, 308); this hypothesis is contradicted by the apparent electroneutrality of phosphate uptake. What is currently known suggests, instead, that respiration leads to expulsion of protons and enhancement of the *p*H gradient. This is limited, however, by the development of a membrane potential: only when a suitable cation is available can net proton extrusion take place. In other words, availability of the cation converts $\Delta\psi$ to ΔpH; phosphate uptake is enhanced, and the salt accumulates in the mitochondrial matrix (Fig. 7b; see references 215, 248, 309).

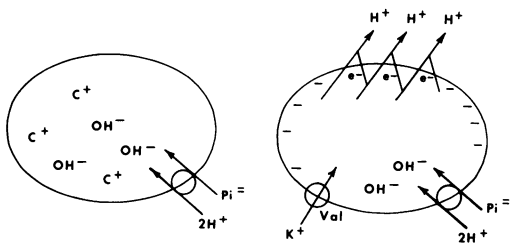

FIG. 7. *Transport of phosphate by mitochondria, via Pi^-/H^+ symport in response to a pH gradient. Left, Nonrespiring mitochondria; right, stimulation of phosphate uptake by respiration, in presence of a cation. Val, Valinomycin.*

The osmotic swelling technique led to the first recognition of not only the phosphate carrier but of transport systems for substrate anions as well (67, 70). These interact in a remarkable manner. Transport of malate requires Pi for "activation" and is now known to occur by exchange of malate for Pi; this is an electrically neutral process which requires no metabolic coupling, since it occurs in mitochondria whose respiration is blocked. Consequently, malate distribution across the membrane reflects that of phosphate and, secondarily, the *p*H gradient. Uptake of citrate, in turn, was found to require "activation" by malate. In fact, citrate uptake occurs by exchange for malate; one proton accompanies citrate, so that once again the overall process is electrically neutral. Like that of other anions, citrate distribution is ultimately linked to the *p*H gradient (162, 248, 296–300, 309).

Overall, then, the uptake of anions by mitochondria—either resting or respiring—is thought to depend upon a cascade of secondary transport catalysts, as shown in Fig. 8. The key element is the phosphate porter, since phosphate uptake is directly coupled to proton movements. Net uptake of malate must be coupled to prior Pi uptake, which thus acts cyclically as a catalyst; citrate uptake, by exchange for malate, is even more remotely linked to the prime mover of the cascade—the *p*H gradient across the membrane. The greatly enhanced uptake of anions, together with cations, is at least qualitatively explained by any scheme which assigns the respiratory chain the function of expelling protons with the generation of a ΔpH and of a membrane potential.

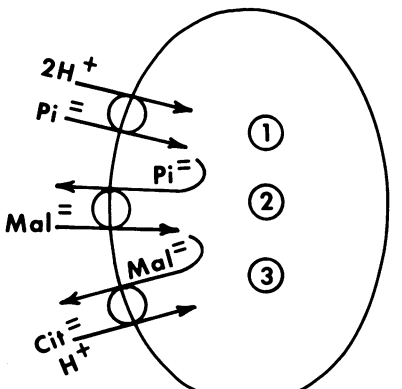

FIG. 8. *Interlocking systems for anion transport in mitochondria. (1) Phosphate-proton symport; (2) malate-phosphate antiport; (3) citrate-malate antiport.*

Summary: Energy Transductions in Mitochondria

A reviewer feels most comfortable when seated firmly on the fence. But it is clearly necessary to conclude this torrent of experiment and argument by stating briefly the upshot of the dispute. The following is inevitably one man's view.

Figure 1 represents schematically the consensus regarding the general pattern of mitochondrial energy transductions. The respiratory chain supports not only the synthesis of ATP but a number of other energy-linked functions, including particularly the transport of ions and substrates. The crucial insight is that these functions do not require prior ATP synthesis but are linked more directly to the chain. Conversely, exogenous ATP or even an ion gradient can sustain other energy transformations. Thus mitochondria, unlike the cell as a whole, do not use ATP as the universal energy currency: ATP is but one of several modes of energy storage, all of which derive from a common pool of energized intermediates or state.

The nature of the energized state continues to be, as it has for a decade, the heart of the dispute, and the devil finds no lack of scriptures to quote for his own purposes. But there is a clearly discernible trend, in that elements that were originally derived from Mitchell's chemiosmotic hypothesis have been assimilated into all the major attempts to understand the molecular basis of energy coupling. Consequently, it no longer seems so urgent or even meaningful to disprove one or another of the original hypotheses *in toto*; rather, the need is for alternative and testable interpretations of the data now at hand.

(i) The mitochondrial membrane is inherently impermeable to H^+ and OH^- and, indeed, to ions generally. Functionally specialized mechanisms exist for the translocation of selected metabolites, which operate in such a manner as to maintain the osmotic equilibrium and, apparently, the gradients of *p*H and of electrical potential.

(ii) Oxidative phosphorylation has never been observed in a preparation that did not contain topologically closed vesicles.

(iii) The respiratory chain contains both hydrogen and electron carriers which appear to be organized both within and across the membrane. The sequence of carriers is still debatable, and at present only one loop (rather than three) can be strung across the membrane. But it is difficult to see what function the arrangement serves other than to separate protons and

electrons across the membrane.

(iv) The balance of the evidence is that the oxidation chain does bring about a separation of electrical charges. Mitochondria can generate an electrical potential of the order of −250 mv, interior negative. In chloroplasts and sonic submitochondrial particles, the polarity is reversed and the interior is positive. Whatever may be the mechanism of charge separation, the very existence of large membrane potentials must be of profound significance to mitochondrial function.

(v) Is the ATPase of mitochondrial and chloroplast membranes reversibly coupled to proton translocation? The data on ion transport indicate strongly that ATP hydrolysis does bring about the electrogenic extrusion of protons, and conversely that ATP synthesis can be driven by an ion gradient. The mechanism of proton extrusion by the ATPase seems an area of darkness. Many data are in principle consistent with Mitchell's proposal that the proton gradient (ΔpH and $\Delta\psi$) reversibly determine the poise of the ATP/ADP couple. Other findings, however, suggest that chemical precursors to ATP do exist.

(vi) Mitochondria accumulate a variety of metabolites—cations, anions, substrates, nucleotides. It is clear that specific transport systems mediate the translocations, but there is no reason to invoke "active transport." Transport of ions and solutes against substantial concentration gradients can be explained quite satisfactorily as secondary responses to the gradient of pH and of the electrical potential. Evidence for the existence of the requisite symport and antiport systems is mounting rapidly; for the present at least, there is no clear evidence for primary energy-linked anion or cation pumps.

(vii) The recognition that certain pharmacological agents exert their effects by serving as artificial ion carriers is one of the most important results of recent membrane research. The ionophores are models for the association of nautral membrane carriers with their substrates and provide tools with which to explore the intimate relationship of energy metabolism to ion movements. Uncoupling seems to result, in many cases, from the conduction of protons or other ions. It is not possible to state conclusively whether uncoupling results from the conduction of protons *across* the membrane, or *into* its hydrophobic region, but a fundamental relationship of uncoupling to ion movements seems to me undeniable.

(viii) And so we come at last to consider the molecular nature of energy conservation. It is clearly possible, as Skulachev (350) has recently done, to formulate reasonable models consistent with at least most of the data in terms of both principal hypotheses. In the chemiosmotic view, the oxidation chain and the ATPase are each seen as entirely separate, primary translocation systems for protons, which are coupled via ΔpH and $\Delta\psi$. According to the chemical hypothesis, the oxidation chain and ATPase each generate an unspecified high-energy intermediate; this in turn is the energy donor for a proton pump that produces ΔpH and $\Delta\psi$. There is much to recommend a constructive synthesis, which includes chemical intermediates on the direct path to ATP, and ΔpH and $\Delta\psi$ as the energy donors for transport. Perhaps a counter-culture (210) deserves the last word:

"Myself when young did eagerly frequent
Doctor and saint, and heard great argument
About it and about, but evermore
Came out by the same door where in I went."

ENERGY TRANSFORMATION IN BACTERIAL MEMBRANES

The cytoplasmic membrane of bacteria performs diverse functions which, in eukaryotic cells, are assigned to specialized organelles. The structure which regulates the metabolic traffic between medium and cytoplasm serves both as osmotic barrier and as osmotic link. It bears the pigments and catalysts of the respiratory chain and provides the structural framework for oxidative phosphorylation. Flagellae are attached to the membrane, which may also provide the energy for motility. And finally, it is the locus of enzymes for the biosynthesis of structures external to the osmotic barrier and an anchor point for the genophore.

Bacteria are profoundly different from mitochondria in structure as well as in function, and it is obvious that one extrapolates from mitochondria to bacteria at some hazard. Yet the fundamental unity of biochemistry makes it exceedingly likely that the basic principles of membrane function will be more or less universal, just as the principles of genetics are universal. In the remainder of this essay we shall examine energy-linked processes in bacterial membranes—oxidative and photosynthetic phosphorylation, motility, and active transport—in the hope that insights derived from the study of organelles may illuminate some of the mysteries of membrane function in bacteria.

Structural Basis

The envelope of bacterial cells consists of a succession of layers, one within the other. That of gram-negative organisms is particularly complex, including two lipoprotein membranes and the periplasmic space between them. The outer, lipopolysaccharide membrane, is usually believed not to constitute a permeability barrier to small molecules. This may be oversimplified, but it is nonetheless true that in all bacteria it is the cytoplasmic membrane proper which carries out the interconversion of chemical, mechanical, and osmotic forms of energy. It appears in most electron micrographs as a plain "unit membrane," whose unremarkable appearance gives no hint of its true complexity.

Autotrophic bacteria characteristically possess elaborate intracellular membranes, but the more familiar heterotrophs do not. Here, the only morphologically differentiated regions that are regularly encountered are the vesicular bodies called mesosomes. These are now known to occur in both gram-positive and gram-negative bacteria (reviewed in 318, 327), but after a decade of research their function remains unclear. At one time it was quite widely held that mesosomes are sites of oxidative metabolism, but this supposition can now be discounted. Recent studies by Frehel et al. (118) demonstrate that deposits of tellurite, which are reliable cytological indicators of respiratory activity, are scattered over the plasma membrane but never occur on the mesosomes. Moreover, isolated mesosomes are deficient in NADH oxidase, succinic dehydrogenase, ATPase, and cytochromes (94, 107, 312, 365, 366). The evidence that mesosomes are involved in cell division, circumstantial as it is, carries more conviction. Attachment of deoxyribonucleic acid to the membrane is often, but not always, mediated by a mesosome. Moreover, the frequent appearance of mesosomes at the site of septum formation points to a role in this process, and perhaps in the synthesis of cell wall or of membrane. The precise nature of this role remains uncertain. The membrane fraction is known to contain enzymes for the biosynthesis of cell wall peptidoglycan, but I am not aware of any evidence as to their presence in mesosomes. The C_{55}-polyisoprenoid alcohol which serves as a glycosyl carrier in wall biosynthesis is about equally distributed between the mesosome and plasma membrane fractions of *Lactobacilli* (366). It also now appears (94), contrary to earlier suggestions, that mesosomes are not sites of preferential synthesis of phospholipids. An attractive speculation is that mesosomes, like the Golgi apparatus of mammalian cells, are involved in the export of macromolecules or building blocks across the membrane. Lampen and his associates (123) have described the association of an apparatus of tubules and vesicles with the secretion of penicillinase by *Bacillus licheniformis*.

Another common morphological feature is the occurrence of stalked particles on the inner surface of the plasma membrane of both gram-positive and gram-negative bacteria (1, 49, 122, 278, 279). These structures contain the Ca^{2+}-activated ATPase which is part of the apparatus of oxidative phosphorylation and are presumably homologous with the stalked particles so characteristic of the inner mitochondrial membrane. They are scattered, apparently at random, over the entire membrane surface. It is curious that the ATPase of the homofermentative organism *Streptococcus faecalis*, which does not carry out oxidative phosphorylation, appears to lack a visible stalk (A. Abrams, *personal communication*).

Further insight into the organization of the cytoplasmic membrane has been obtained by means of chemical and physical methods. It now appears that the prolonged conflict over the essential nature of membrane structure (see Hendler, 153, for an excellent summary) is begining to subside. Davson and Danielli, some 40 years ago, proposed that large regions of biological membranes are organized into a bilayer of phospholipid molecules: The polar headgroups face the medium and the fatty acyl side chains make up a liquid, hydrophobic phase in the interior of the membrane which constitutes the permeability barrier to ions and polar molecules. Despite widespread dissent just a few years ago, it now appears that this classical model provides an essentially correct description of the membrane as a barrier. Much of the evidence derives from the examination of *Mycoplasma* membranes by a variety of physical techniques (57, 95, 97, 357, 368, 391). The properties of ion-conducting antibiotics are consistent with this model (277) as are various experiments which indicate that the lipid elements of the membrane exist in a semiliquid state (291, 369).

On the other hand, students of membrane functions have long insisted on the essential role of proteins in the organization of the cytoplasmic membrane, a role not adequately acknowledged by the classical "picket fence." Here again the study of *Mycoplasma* membranes has been fruitful. Chemical dissection (276) suggests that the bulk of the membrane

proteins are associated with the surface, but there is a fraction which is imbedded in the interior. Freeze-etching of *Mycoplasma* membranes (368) reveals particles which may be protein molecules buried in, or even plugged through, the membrane in specific regions. The physical properties of the bulk lipids suggest that regions in which the lipids associate with the proteins are quite limited in extent.

In this connection, it is worth stressing that analysis of the proteins of bacterial membranes by gel electrophoresis reveals several dozen components but no single, major constituent to correspond to the expectation of a "structural protein" (71, 254, 324, 330, 331, but also 302). The evidence on which the concept of a structural protein in mitochondrial membranes was originally based is now known to be faulty (reviews: 197, 310); proteins whose sole function is structural may well exist in membranes, but the idea now has little experimental foundation.

There emerges the impression that the bacterial plasma membrane is a mosaic, composed of small functional regions that are predominantly protein embedded in a relatively homogeneous bilayer matrix. Hydrophobic interactions between these constituents predominate over ionic ones and the phospholipid headgroups may be largely exposed at the surface. The lipid bilayer must remain intact if the membrane is to serve as a permeability barrier: this inference, whose chief support was research on antibiotics and drugs which interact with membranes (review: 132), was given a firm foundation by Kaback in his studies on the role of lipids in bacterial membrane vesicles (reviews: 186–188). Bulk lipids, such as phosphatidylethanolamine in *Escherichia coli*, are essential components of the barrier. Functional membrane proteins are associated with the lipid phase in varying degrees of intimacy. Some, including the stalked ATPase, can be dissociated from the membrane by gentle washing and then reattached to restore a functional complex (4, 6, 26). At the other extreme, the M protein of the β-galactoside transport system can be extracted only with difficulty and detergents, and probably requires lipid for its activity. Indeed, many membrane proteins are not known to require lipids—in some cases specific lipids. Examples include NADH dehydrogenase (280) and the phosphotransferase system which catalyzes the vectorial phosphorylation of glucose (228, 253, 321). The role of lipids in the functioning of some bacterial membrane enzymes has been reviewed (323). Virtually nothing appears to be presently known regarding the spatial relationship of functional centers to each other and to the respiratory chains.

One of the most intriguing consequences of the essentially liquid nature of membranes is their ability to reseal after mechanical injury. This fact, well known to cell physiologists since the thirties, was first applied to the study of erythrocytes which can heal after lysis to form vesicles having the orientation of the original cell. By judicious control of conditions, one may produce at will both normal and inverted vesicles (355, 356). By the same token, as was discussed above, disruption of mitochondria can lead to the production of both kinds of vesicles.

Membrane vesicles from bacteria have begun to attract attention only recently, under the impact of the work of H. R. Kaback, but are certain to assume increasing prominence in the near future. To facilitate discussion of the origin and orientation of such particles, Fig. 9 illustrates the various vesicles that can be expected to arise by disruption of the cytoplasmic membrane; the stalked particles that contain the ATPase (*see below*) mark the cytoplasmic side. The nature of "fragments," i.e., topologically open structures, and of "right-side-out" and "inside-out" vesicles requires no elaboration. "Patchwork" vesicles could arise by the annealing of fragments differing in orientation, so that the marker faces out in some patches, inward in others. Tsukagoshi and Fox (369) recently reported the formation of hybrid

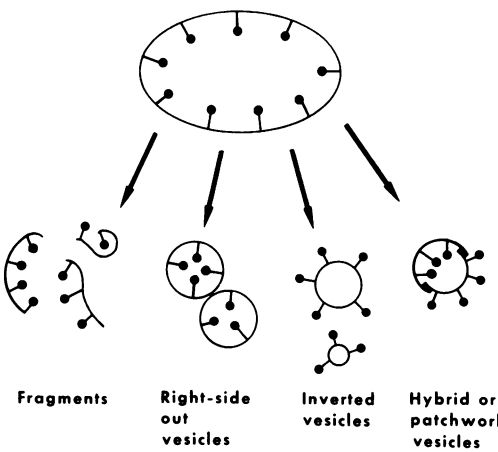

FIG. 9. *Formation and orientation of membrane vesicles. The stalked adenosine triphosphatase particles are used as markers for the cytoplasmic side of the membrane.*

vesicles when a mixture of membranes of different origins was subjected to sonic treatment, and some of these may have the "patchwork" structure. It is also conceivable that a marker protein could flip over during preparation of the vesicles. The last possibility raises a disturbing question: Is it conceivable that membrane proteins (apart from circulating carriers) could shift reversibly in a given vesicle, so as to face first one side and then the other, without impairing the topological integrity of the vesicle? If this possibility is admitted, the concept of sidedness becomes indeterminate, at least for that particular protein.

Oxidative Phosphorylation

General features of respiration and phosphorylation. In the bacterial respiratory chains, as in that of mitochondria, reducing equivalents originating in NADH, succinate, or other electron donors pass over a cascade of carriers to oxygen. Various aspects of bacterial respiratory chains are covered in recent reviews (49, 122, 168, 353, 390), all of which stress features in which the respiratory chains of bacteria differ from each other and from those of mitochondria. The essential point is that bacterial respiratory chains are more flexible: They oxidize a wider range of substrates than mitochondria do, and in some cases at least can employ sulfate or nitrate as terminal electron acceptors in place of oxygen. Bacterial respiratory chains often appear to be branched (390), consisting of several distinct chains which communicate at particular sites. Often one finds constituents unique to bacteria, as among the cytochromes. Some bacteria contain ubiquinones, others menaquinones (related to vitamin K), and some contain both.

Important as such differences are, there is no reason to doubt that the coupling of respiration to phosphorylation is in principle the same in bacteria as in mitochondria (49, 122). The present discussion, restricted to selected organisms which will be considered repeatedly in this article, is intended only to summarize the main features of the architecture of the respiratory chain and its overall metabolic performance.

(a) *Escherichia coli* inevitably heads the list. This is one of the most versatile of bacteria, capable of growing anaerobically either by glycolysis or by the use of nitrate as terminal electron acceptor. The enzymatic machinery of oxidative phosphorylation is produced only under aerobic conditions. In addition, it may not always be appreciated that expression of the capacity for oxidative phosphorylation is repressed by glucose (63, 149, 328).

Oxidative phosphorylation supplies at least a large fraction of the ATP of aerobic cells of *E. coli* and related organisms, since the level declines if the cells are made anaerobic or exposed to uncouplers. Studies with intact cells suggest the generation of 3 moles of adenosine triphosphate for each NADH oxidized, for a total of three coupling sites in this pathway (75, 145, 148).

E. coli contains both a benzoquinone (coenzyme Q) and a naphthoquinone (vitamin K_2). The isolation of mutants blocked in the synthesis of one or the other of these has contributed greatly to the analysis of the role of quinones. It seems clear that coenzyme Q is the quinone chiefly involved in the respiratory chain—at least, in the oxidation of NADH, malate, and lactate. Oxidation of these substrates was impaired in a ubiquinone-deficient mutant, and glucose was converted to lactic acid (79). Studies with these mutants and with inhibitors of the respiratory chain (79, 354) suggest the chain shown in Fig. 10, with coenzyme Q playing a vital but possibly indirect role in electron transport. The role of menaquinone appears to be much more restricted: it serves as a hydrogen carrier in a particular step in uracil biosynthesis (283). (It should perhaps be mentioned that in *Proteus*, Kröger et al. [227] place coenzyme Q in the direct pathway of electron transport but suggest that menaquinone serves as an alternative carrier of reducing equivalents in the oxidation of NADH). There is no information at present as to the orientation of the respiratory chain within and across the membrane, but it is curious that Hadjipetrou et al. (129) find inhibition of respiration by ferricyanide; this would not be expected to pass across the membrane and may perhaps be functioning as an unnatural electron acceptor at some exterior site.

(b) *Micrococcus denitrificans* is another adaptable organism. It grows heterotrophically with oxygen as terminal acceptor; anaerobically, nitrate or nitrite can substitute for oxygen, and it can even be grown autotrophically on hydrogen gas under appropriate conditions. The respiratory chain, so far as it is known (Fig. 10), is remarkably similar to that of mammalian mitochondria except for branching at the oxygen terminus (15, 169, 170, 217, 339). The response of the chain to inhibitors is also similar to that of mitochondria and defines three possible coupling sites. Studies with in-

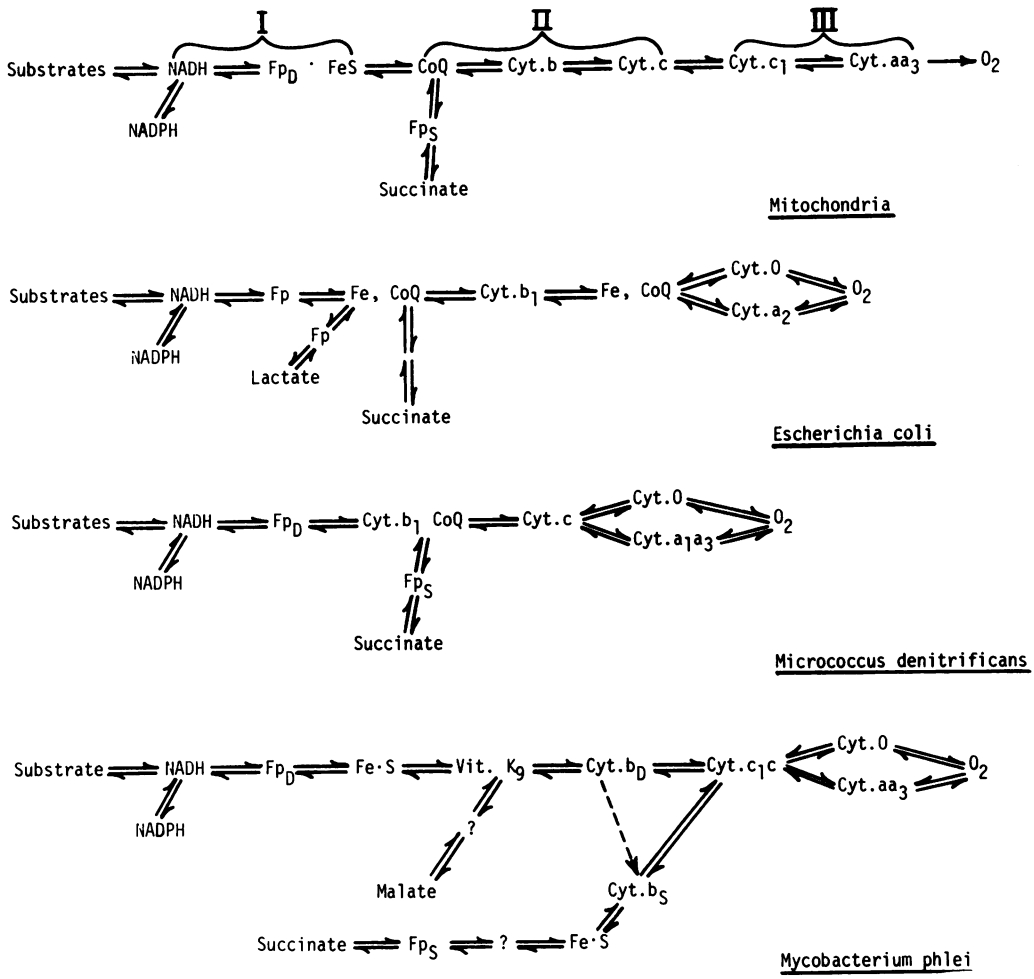

FIG. 10. *Respiratory chains of mitochondria and selected bacteria. Fp, Flavoproteins; CoQ, coenzyme Q; FeS, nonheme iron. For references see text.*

tact cells, however, suggest that there may be as many as four (336). One of these may correspond to the transhydrogenase site first described by Asano et al. (15).

There is a hint that at least part of the chain may be oriented across the membrane, since even intact spheroplasts can oxidize exogenous ferrocytochrome *c* (339). It is curious that cytochrome *c* can be partly released from intact cells by washing with salt solutions (334), but it remains to be seen whether this reflects the position of cytochrome *c* at the external surface of the membrane, as appears to be the case in mitochondria.

(c) *Mycobacterium phlei* is a strict aerobe. It has been studied in detail by Brodie and his associates, who have undertaken the arduous task of dissociating and reassembling the components of the oxidative phosphorylation apparatus. A detailed review has been prepared by Brodie and Gutnick (49).

Present knowledge of the respiratory process, with its three distinct chains leading from NADH, succinate, and malate, is summarized in Fig. 10 (after references 13, 49; and A. F. Brodie, *personal communication.*) The general similarity of this chain, with three coupling sites and transhydrogenase, to that of mitochondria is evident. There is, however, one notable exception: the natural quinone in *M. phlei*, as in gram-positive organisms generally, is a naphthoquinone related to vitamin K.

It is clear that vitamin K is intimately and specifically involved in respiration coupled to phosphorylation, but its precise role is uncertain. It has been proposed that a phosphoryl-

ated derivative of vitamin K is formed during electron transport and donates its phosphoryl group to ADP; this, if true, would imply a mechanism radically different from the mitochondrial one, since the energy-rich intermediate or state in these organelles is almost certainly not phosphorylated. Gutnick and Brodie (128) detected incorporation of tritium into the naphthoquinone of the respiratory chain under conditions when oxidative phosphorylation is taking place; this incorporation was sensitive to uncouplers. Subsequently, Watanabe and Brodie (382) isolated a phosphorylated derivative of vitamin K when membrane fragments were incubated with excess vitamin K as electron sink; whether this, or a related compound, does indeed serve as the natural phosphoryl donor for ATP synthesis remains to be demonstrated.

In this context it should be mentioned that in *Bacillus megaterium*, in which a vitamin K is again the natural quinone, Kröger and Dadak (226) find no evidence for participation of the naphthoquinone in phosphorylation. They suggest that it functions, as does ubiquinone in mitochondria, to collect reducing equivalents from various dehydrogenases for delivery to the cytochromes.

(d) *Streptococcus faecalis*. Until recently it seemed safe to regard streptococci as purely fermentative organisms. Lacking cytochromes and oxidative phosphorylation, the only known pathways for the generation of metabolic energy were substrate-level phosphorylations such as glycolysis, the phosphoclastic cleavage of pyruvate, and the catabolism of arginine. The flavoprotein-linked NADH oxidase was generally regarded as not coupled to energy production.

This simple pattern must now be qualified. Growth yields of aerobic cultures first pointed to the occurrence of limited oxidative phosphorylation. This has now been confirmed with membrane preparations which do appear to couple NADH oxidation to ATP synthesis, albeit with low efficiency (105, 252). Results obtained with fumarate as an artificial electron acceptor suggest a chain corresponding to the first coupling site of mitochondria (Fig. 10). Oddly, the system was inhibited by antimycin, which is believed to act at the level of cytochrome *b*, but was unaffected by an uncoupler. To complicate matters further, it now appears that at least one strain of *S. faecalis* does form cytochromes when grown on a medium supplemented with hematin. Phosphorylating membrane particles were again obtained (52).

Coupling factors: the role of ATPase. During the past decade, phosphorylating membrane particles have been prepared from a variety of bacterial species. The low efficiency of oxidative phosphorylation by such particles, whose P/O ratios are generally below one, was at first taken to suggest that oxidative phosphorylation in bacteria is inherently less efficient than in mitochondria. But now it is generally recognized that intact bacterial cells have P/O ratios quite comparable to those of organelles, and that the low efficiency of particles reflects structural damage and the loss of components. Soluble "coupling factors" were found to be required for oxidation, phosphorylation, or both; P/O ratios greater than one, or even two, have been obtained from several systems including preparations from *M. phlei*, *M. denitrificans*, *Azotobacter vinelandii*, and *Alkaligenes faecalis* (review: 49).

The nature, functions, and interrelationships of these coupling factors are far from clear. *M. phlei* particles, for example, require several factors—some soluble, some easily dissociated from the particles by urea (35, 154). However, brief heating of the particles or exposure to trypsin relieved the need for soluble factors and increased the P/O ratio (11, 33, 34). Some of the factors may play a structural role, or else exert their effects in an indirect manner. *Alkaligenes faecalis* extracts require two coupling factors whose proper attachment depends upon Mg^{2+} and K^{+}. One of these, once thought to be a nucleic acid, now appears to be a protein (8–10).

One factor which appears to be common to all phosphorylating preparations is one that exhibits latent ATPase activity. The hydrolytic activity can be unmasked either by trypsin treatment or by heat (10, 32, 33, 172, 173). The resulting ATPase characteristically requires either Ca^{2+} or Mg^{2+} and is cold-labile. Activation results in loss of coupling activity. The soluble factor consists of 9-nm globules (10), thought to correspond to the stalked particles that are so often seen on the inner surface of membranes from aerobic bacteria (1, 10, 49, 122, 278, 279). It seems almost self evident that this enzyme, like the analogous protein in mitochondria and chloroplasts, catalyzes the terminal step in oxidative phosphorylation. Indeed, Bogin et al. (32) have shown that coupling factors of mammalian and bacterial origin can replace each other. Direct evidence for the participation of ATPase in oxidative phosphorylation comes from the recent isolation of a mutant of *E. coli* which is unable to couple phosphorylation to oxidation; membrane fragments and whole extracts of this

mutant had only traces of ATPase activity. The authors tentatively propose that the gene in question is the structural gene for ATPase (54).

Thus far, the ATPase activity of preparations which exhibit coupling factor activity has always been found to be latent. Presumably, as with the enzymes from mitochondria and chloroplasts, the hydrolytic activity is an artefact: in the native enzyme, the structure of the complex may exclude water and favor the transphosphorylation step. The nature of the unmasking is not known; in mitochondria, removal of an inhibitory protein is required, whereas a relatively small configurational change suffices to reveal latent ATPase in chloroplast coupling factor (100, 101).

ATPase activity has been found to be associated with membranes from many bacterial species, and the presence of so potent an enzyme catalyzing a "forbidden" and potentially harmful reaction raises a host of questions. Many preparations exhibit considerable activity without the need for any unmasking (for example 6, 98, 99, 127*a*, 127*b*, 137, 254*a*, 363*a*, and others cited in these references). Is such activity necessarily an artefact reflecting the unphysiological environment of a catalyst whose real function is transphosphorylation? Is there only a single species of ATPase, or are there several? Is ATPase, latent or exposed, necessarily associated with oxidative phosphorylation? In this context, it should be recalled that ATPase occurs in *E. coli* grown either aerobically or anaerobically (54, 98, 99) and in *S. faecalis* which does not ordinarily derive metabolic energy from oxidative phosphorylation (4–6, 137, 332, 333). In both these cases, and in others as well, there appears to be only a single major ATPase species as judged by purification, inhibition studies, and genetic findings. It will be of great interest to determine whether, and how, the ATPase activity is regulated and whether consistent differences can be found between enzymes from glycolyzing and respiring cells.

It seems likely that ATPase enzymes serve as links between cytoplasmic and membrane functions in both directions: on the one hand, to couple the membrane-bound catalysts of oxidative phosphorylation to the synthesis of ATP and, on the other, to enable cells to utilize ATP as an energy source for membrane functions. The mutant of *E. coli*, recently isolated by Butlin et al. (54), which is almost devoid of ATPase activity will surely prove to be of great value in defining the precise role of ATPase. It is already clear that this mutant can neither carry out oxidative phosphorylation (54) nor use ATP to derive the energy-linked transhydrogenation reaction (78). It can grow on glucose as sole source of energy, but not on succinate or lactate. There is at this time no evidence that bears on Mitchell's view (265) that the equilibrium of bacterial ATPase is poised by the proton-motive force. Indeed, evidence for participation of this enzyme in proton extrusion is as yet limited (141, 337).

An important clue to the function of bacterial ATPase enzymes is their striking resemblance to ATPase enzymes of mitochondrial and chloroplast membranes (62, 137, 333). Not only are they alike in general size and shape, but they share such attributes as lability when stored in the cold and sensitivity to DCCD. They require Mg^{2+} (and/or Ca^{2+}) but are not particularly stimulated by Na^+ and K^+ (Table 2). These enzymes are evidently quite unlike the ouabain-sensitive, Na^+-K^+ transport ATPase so characteristic of mammalian plasma membranes. It appears that the latter enzyme is not present in bacteria, even though a trace of activity has been reported (130).

Nature of phosphorylating particles from bacterial membranes. Particles arising by comminution of the bacterial membrane are finding extensive use in the characterization of oxidative phosphorylation, but little attention has yet been paid to their topology. If the conclusions drawn from the work with mitochondria are correct, particles which carry out oxidative phosphorylation must, *ipso facto*, be closed vesicles. Moreover, they cannot be grossly leaky to ions, since it appears that massive ion movements result in uncoupling. Now mitochondria are known to have an ATP/ADP porter, but bacteria presumably do not: what evidence is currently available suggests that bacterial membranes are generally virtually impermeable to ATP, ADP, NADH, NADPH, and other central metabolites. How, then, can one obtain particles which are topologically closed yet accept NADH and ADP? The conclusion seems inescapable that such particles are inside-out, so that the coupling device faces the medium (260).

Of course, membrane preparations may well contain several kinds of vesicles as well as open fragments, and interpretation of their activities must take this heterogeneity into account. For example, if we assume that NADH dehydrogenase is localized at the inner surface, then the rate of NADH oxidation will reflect nonphosphorylative oxidation by open fragments and coupled oxidation by inverted vesicles, but may not include that fraction of

TABLE 2. *Comparison of ATPases from various sources*

Characteristic	*Streptococcus faecalis*	*Escherichia coli*	*Micrococcus lysodeikticus*	Rat liver mitochondria	Na^+, K^+ transport ATPase (mammalian plasma membranes)
Molecular data	MW[a] = 385,000 S_w = 13.4 S Subunits: 6 pairs	MW > 100,000	MW ? S_w = 14–15 S Subunit no. not known	MW = 384,000 S_w = 12.1 Three classes of subunits	MW = 250,000 to 500,000; subunits uncertain
Morphology	Hexagon, about 10-nm diam; no clear stalk, but needs nectin for attachment.		Stalked particle	Stalked particle, about 10-nm diam	
Stability and latency	Stable when bound, soluble enzyme cold-labile; no activation needed	Stable when bound, soluble enzyme cold-labile; no activation needed	Soluble enzyme cold-labile (?); activated by trypsin	Soluble enzyme cold-labile; activated by trypsin	Phospholipids required for activity
Phosphorylated intermediates	Apparently none			Apparently none	$ATP \xrightarrow{Na^+} ADP + E \sim P$ $E \sim P \xrightarrow{K^+} E + Pi$
Activation by cations	Mg^{2+} required; Na^+ and K^+ activate slightly at high concn	Mg^{2+} or Ca^{2+} required; no effect of Na^+, K^+	Mg^+ or Ca^+ required; little effect of Na^+, K^+	Mg^{2+} required; little effect of Na^+, K^+	Mg^{2+}, Na^+, and K^+ required for maximal activity
Inhibitors	DCCD, Dio 9, guanidines; no inhibition by oligomycin or ouabain	DCCD, azide; no inhibition by oligomycin or ouabain	Azide; no inhibition by oligomycin or ouabain	Azide, guanidines, DCCD, Dio 9, oligomycin; no inhibition by ouabain	Very sensitive to ouabain; high levels of oligomycin inhibit, little effect of DCCD
References	4, 5, 26, 137, 138, 332, 333	54, 98, 99	172, 173, 278, 279	62; also 310, 343, 375	76, 348

[a] MW, Molecular weight.

the enzyme activity sequestered in structures that are right-side out. Thus, topological factors may be responsible, at least in part, for the apparent existence of both phosphorylating and nonphosphorylating pathways of oxidation and other puzzling observations.

The above is, admittedly, an *a priori* argument. However, a body of circumstantial evidence bearing on the orientation of membrane vesicles is scattered through the literature. Some of the arguments turn on the properties of ionophores and lipid-soluble ions, which are summarized in Table 1 for quick reference.

(a) *Micrococcus denitrificans*. Scholes and Smith (338) described the preparation of membrane vesicles by osmotic lysis of spheroplasts. Like the original spheroplasts, the membranes oxidized succinate and NADH slowly, but both activities were enhanced by repeated freezing and thawing. Oxidation of succinate by membranes was also stimulated by the nonionic detergent Triton X-100, whereas the activity of sonically treated membranes was not. The authors proposed that their membrane preparation consisted largely of intact vesicles of the same orientation as the intact cell (i.e., right-side-out).

Using a slightly different procedure of lysing spheroplasts, John and Whatley (183) and John and Hamilton (181, 182) prepared particles which couple oxidation of NADH to phosphorylation with high efficiency, and exhibit respiratory control: ADP, and also the uncoupler CCCP, stimulated respiration. Like submitochondrial particles, the *M. denitrificans* particles could also be uncoupled by a combination of valinomycin, K^+, and nigericin. We shall return to the significance of these observations with ionophores in the following section. For the present we shall only note the conclusion (181, 182) that the bacterial particles are closed vesicles and inside-out.

(b) *Mycobacterium phlei*. As mentioned above, detailed studies have been carried out on particulate membrane preparations of *M. phlei* which perform oxidative phosphorylation with relatively high efficiency (P/O ratios about two for NADH and succinate). Several results suggest that the process depends upon intact vesicles, especially the observation that repeated freezing and thawing abolished oxidative phosphorylation, but brief treatment at 50 C restored it (11, 34).

Very recently it has become apparent that at least two kinds of particles can be generated from *M. phlei* (14). Protoplast ghosts contain all the components necessary but carried out very little oxidative phosphorylation; prolonged preincubation with substrate and ADP was required, and the ATP formed was at least partly retained. Oxidative phosphorylation by these ghosts, like that by intact mitochondria, was uncoupled by valinomycin plus K^+ and by dibenzyldimethyl ammonium (DDA^+). Thus the protoplast ghosts appear to have the same orientations as do the parent cells. Sonic treatment produced much more active preparations which appear, by all available criteria, to be inverted. NADH oxidation and P/O ratios were greatly increased; sonic particles were not uncoupled by valinomycin plus K^+, or by DDA^+, but only by valinomycin plus nigericin, or by the lipid-soluble anion tetraphenylboron; stalked particles corresponding to the latent ATPase were observed facing the medium. All this strongly suggests that oxidative phosphorylation by membrane fragments requires inverted vesicles. By contrast, accumulation of proline at the expense of respiration (157) is apparently characteristic of vesicles that are right-side-out.

(c) *Escherichia coli*. Of all bacterial membrane vesicles, those prepared from *E. coli* by Kaback are by far the best characterized. Several lines of evidence indicate that vesicles, prepared by osmotic lysis of spheroplasts according to a carefully specified procedure, are topologically closed and have the same orientation as do the original cells. Among the criteria employed are negative staining in electron micrographs, the reduction in internal space as a function of the osmotic pressure, retention of colloidal gold, and others (185, 188). Such vesicles accumulate sugars, amino acids, and other substances by a process linked to the oxidation chain, which will be considered in detail below. For the present, let us note that such vesicles do not appear to carry out oxidative phosphorylation (43, 188, 213). Whether this is due to the loss of essential factors or is simply a consequence of their orientation remains to be determined.

Particulate preparations of *E. coli* have been described which do carry out oxidative phosphorylation, albeit with low efficiency (44; see reference 49 for earlier preparations). Particular attention has been devoted to the energy-linked transhydrogenase activity of these particles (44, 112, 363). These particles, prepared by sonic treatment, must be inverted, but direct evidence on their polarity has not been reported.

(d) *Streptococcus faecalis*. Gentle lysis of protoplasts by dilution in a medium of low osmotic strength yields vesicles which have low ATPase activity and appear to be right-

side-out. Sonic treatment reveals the ATPase, apparently by fragmentation (Altendorf and Harold, *unpublished data*).

In summary, the principle that bacterial membrane preparations can reseal to give vesicles that are either right-side-out or inverted is an increasingly plausible one. To a first approximation, it appears that gentle lysis of protoplasts is likely to yield right-side-out vesicles, sonic treatment inside-out vesicles. However, there are already enough exceptions to testify that factors other than mechanical ones play a role in determining the orientation of the vesicles. Some of the factors that may apply to erythrocytes have been considered by Steck and his associates (355, 356).

Coupling of respiration to phosphorylation. The labors of many investigators of mitochondria lead eventually to formulation of the scheme shown in Fig. 1. According to this, the redox reactions of the oxidation chain are coupled to ATP synthesis indirectly, via one or more *nonphosphorylated*, energy-rich intermediates or states. The nature of this entity is, as we saw in the first section, controversial—but its existence is not. It is *a priori* likely that so fundamental a relationship will be much the same in bacteria as in mitochondria, but direct evidence bearing on the validity of Fig. 1 for bacterial membranes is as yet meager.

The burden of the argument is, in effect, to demonstrate the essential identity of events in bacterial and mitochondrial membranes. Thus far, the following energy-linked reactions have been studied, in addition to ATP synthesis by oxidative phosphorylation and, of course, transport.

(i) *Reversed electron transport*. Reduction of NAD by succinate in presence of ATP has been described in several organisms and examined in some detail in *M. denitrificans* (16) and in *E. coli* (362). As in mitochondria, this uncoupler-sensitive process is thought to imply reversal of the first coupling site. Its physiological significance in heterotrophs is uncertain, but in photosynthetic bacteria and in chemoautotrophs it is likely to be a major source of reduced pyridine nucleotides (378, 381).

(ii) *Transhydrogenase*. The strongest evidence for a nonphosphorylated energy-rich intermediate comes from the energy-dependent reduction of NADP by NADH by membrane particles (15, 44, 78, 112, 363). Particles from *E. coli* fully adapted to oxidative phosphorylation could couple transhydrogenation either to the oxidation of succinate or to the hydrolysis of ATP. When ATP served as the energy source, transhydrogenation was inhibited by DCCD and also by uncouplers; when succinate was the energy donor, the process was sensitive to uncouplers but not to inhibitors acting at the level of ATPase. Particles from cells that had been grown anaerobically could use ATP as an energy donor, but not succinate (112). A mutant deficient in ATPase was unable to couple transhydrogenase to the utilization of ATP, though activity with succinate was normal (78). These results point squarely to the existence of two distinct pathways, linked via a common, "energized" intermediate as required by Fig. 1.

As to the actual mechanism of coupling, i.e., the nature of the energized state or intermediate designated "~," there is as yet little information from bacterial systems. However, a recent series of studies with *M. denitrificans* (335, 336) suggests that, as in mitochondria, oxidative phosphorylation requires generation of a proton-motive force across the membrane. Intact cells are virtually impermeable to H^+, but the proton conductance was greatly increased by FCCP (the fluorinated analogue of CCCP [Fig. 5]); this also stimulated respiration. Respiration was associated with ejection of protons from the cells. Maximal proton ejection was observed in the presence of mobile ions—either SCN^- or K^+ together with valinomycin. Under these conditions, eight protons were ejected per oxygen atom, and the rate of respiration (and proton ejection) was comparable to that seen in presence of FCCP. The uncoupler accelerated the return of protons into the cells, but did not affect the H^+/O ratio. From these and other results, Scholes and Mitchell (335, 336) concluded that respiration of *M. denitrificans* is associated with translocation of protons outward, generating a pH gradient and membrane potential. To account for the results it is necessary to postulate an oxidation chain of four loops; one of these may be the transhydrogenase. While no final claim was made, the results are compatible with a chemiosmotic mechanism of energy coupling.

Vesicles of *M. denitrificans*, which are thought to have an orientation opposite to that of the intact cells, were examined by John and Hamilton (181, 182). The particles had a P/O ratio of 1.3 and, unlike most bacterial preparations described in the literature, exhibited respiratory control. The crux of the argument is that respiration was uncoupled by FCCP, by valinomycin plus NH_4^+ and by the combination of valinomycin K^+, and nigericin. These particular combinations characteristically uncouple submitochondrial particles (but not in-

tact mitochondria) and point to the importance of an electrical potential, interior *positive*, across these inverted membranes. (Let me reason out one of these, to clarify the basis of the conclusion. The respiratory chain in these inverted vesicles would translocate protons inward, generating a positive potential. Nigericin allows the H^+ to exchange for K^+, dissipating the ΔpH but not $\Delta\psi$. Valinomycin permits the K^+ to flow out, thus collapsing $\Delta\psi$ as well. Thus, instead of a proton motive force which could reverse the poise of the ATPase, there is a cyclic flux of K^+ through the system) This conclusion is consistent with the observation (64) that membrane particles from *M. denitrificans*, like submitochondrial particles, enhance the fluorescence of ANS^- when allowed to respire.

Parallel observations are beginning to appear from studies on *M. phlei* (14, 157) and on *Staphylococcus* (180, 286). Skulachev (350) refers to experiments which show that respiring vesicles of *M. lysodeikticus* accumulate lipid-soluble anions, indicating again the generation of a positive potential.

Many questions must be raised concerning the apparent relationship between proton-motive force, membrane potential, and oxidative phosphorylation. It is not certain at this stage whether the proton-motive force is a prerequisite for ATP synthesis or an alternative form of energy utilization. It is not even established that ATP as well as all coupling sites give rise to a common form of energy storage, nor that all of the energy-linked functions of bacterial membranes are reversibly interconnected as the scheme of Fig. 1 suggests. The role and mechanism of action of the ATPase has scarcely been explored. It would not be surprising if the organization of the processes which conserve and convert energy in bacterial membranes were found to differ in some respects from that of mitochondria. But it is unmistakeably clear that microbiologists can no longer ignore the controversies that have agitated their mitochondrial colleagues for the past decade.

Photosynthetic Phosphorylation

A small number of bacterial genera share with the higher plants the capacity to utilize light energy for growth. Perhaps for this reason, the investigation of bacterial photosynthesis has taken place largely within the framework of photosynthesis research rather than that of microbial physiology. To do justice to this sophisticated topic within the confines of the present general survey seems to me impossible; this section serves only to relate bacterial photosynthesis to other energy conversions that occur in bacterial membranes. This cavalier treatment is excused by the existence of several current and authoritative reviews (23, 81, 119, 150, 294. 342, 378, 380).

In intact bacterial cells, the photosynthetic apparatus appears as a system of closed sacs, vesicles, or lamellae. Whether the mature structures are continuous with the plasma membrane seems to be still uncertain. In any event, the chromatophores which can be isolated after comminution of the native apparatus are clearly a collection of closed vesicles. They contain bacterial chlorophyll and a variety of carriers for both hydrogen and electrons, and serve the same metabolic functions as do the chloroplasts in plants: the generation of ATP and of reducing power for biosynthesis.

The overall process of bacterial photosynthesis as exemplified by *Rhodospirillum rubrum* is shown schematically in Fig. 11. The initial event is the photochemical excitation of particular chlorophyll molecules localized in a light-harvesting reaction center. In consequence, an electron is transferred to an unidentified primary acceptor, and the chlorophyll accepts an electron from cytochrome c_2.

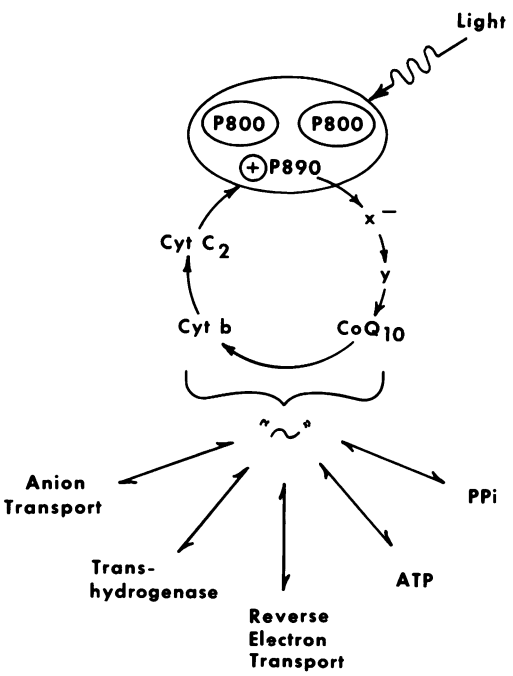

FIG. 11. *Photosynthesis: electron transport and energy transformations in Rhodospirillum rubrum. After Vernon (378) and Frenkel (190).*

Between the unidentified acceptor and the cytochrome extends a chain of redox carriers whose nature, number, and sequence are debatable. This cyclic oxidation chain is coupled to phosphorylation at several sites—most probably two; the end product may be ATP or inorganic pyrophosphate.

Alternatively, light energy can be utilized for the generation of reducing power. Unlike green plants, which possess an additional photochemical system, the bacteria are unable to use water as a hydrogen donor but require an exogenous reducing agent: H_2, H_2S, or an organic reductant. Formation of NADH from NAD and a reductant is envisaged to occur by a process analogous to the reversal of the mitochondrial oxidation chain discussed above. It should be noted that either light or ATP can serve as the energy donor (174, 204, 216).

Apart from the unique photochemical events, the general arrangement of energy transductions appears to be identical with that of oxidative phosphorylation (Fig. 1). The passage of electrons over a cascade of redox carriers is believed to generate an energized state or intermediate, which in turn drives the energy-linked functions: reduction of NAD, transhydrogenase (205), ANS^- fluorescence (18), and the synthesis of ATP or pyrophosphate. All these processes are inhibited by uncouplers which do not, however, interfere with electron transport *per se*. The coupling to ATP synthesis is mediated by a coupling device which includes an ATPase sensitive to both oligomycin and Dio 9 and which requires phospholipids for activity. The ATPase is not, however, involved in electron transport *per se* (21, 40, 113, 214, 251, 337).

A close relationship between photosynthetic phosphorylation and the translocation of protons was experimentally demonstrated by the discovery (23, 379) that isolated chromatophores accumulate protons from the medium upon illumination. Addition of ATP likewise results in uptake of protons (260, 337). Conversely, illumination of intact cells of *R. rubrum* results in extrusion of protons and acidification of the medium (91, 92, 337). It therefore appears that chromatophores are vesicles that have inverted in the course of preparation, so that their polarity is opposite to that of intact cells.

What is the nature of proton accumulation by chromatophores? There is now much evidence to support the thesis that illumination induces rapid, electrogenic H^+ influx, generating across the membrane both a ΔpH (interior acid) and a substantial membrane potential (+250 mv or more, interior positive). This was initially inferred primarily from the effects of ionophores on proton movements and photophosphorylation (176, 202, 285). Recently, Isaev et al. (171) reported that illumination supports the accumulation of synthetic lipid-soluble anions, a clear indication of the development of a positive potential. In addition, Jackson and Crofts (175) showed that the shift of carotenoid spectra upon illumination, which has been known for many years, is a consequence and a quantitative indicator of the membrane potential. Collapse of the gradient of *p*H and of electrical potential by certain combinations of ionophores appears to account for the uncoupling of photophosphorylation (176, 273, 285).

As is required by the scheme illustrated in Fig. 1, ATP and even pyrophosphate serve as energy donors to support membrane functions in the dark: addition of ATP can elicit proton uptake, ANS^- fluorescence, accumulation of anions, transhydrogenase, and the shift in carotenoid spectrum (18, 23, 171, 175, 205, 337). Inhibition of these effects by oligomycin implicates the ATPase of the chromatophore membrane in the utilization of ATP (like the mitochondrial enzyme, chromatophore ATPase is inhibited by oligomycin, whereas other bacterial ATP hydrolyzing enzymes are not). In addition, pyrophosphate can drive the generation of ATP in the dark, again by an oligomycin-sensitive process (203).

As with oxidative phosphorylation, the bone of contention is the precise relationship between proton movements and the primary energy conservation step. According to the chemiosmotic hypothesis, the primary event is the generation of an electrical potential upon illumination. If the acceptor of electrons from chlorophyll and the electron donor are located on opposite sides of the chromatophore membrane, then illumination would not only reduce the one and oxidize the other but also produce a separation of charges across the membrane. An appropriate arrangement of redox carriers would transport H^+ into the chromatophore, conserving the electrical energy and converting part of it into a *p*H gradient (81, 257). However, it is not now possible to rigorously exclude the alternative view that proton uptake and the membrane potential are due to a "proton pump," actuated by energy-rich intermediates or conformations of the membrane produced during electron transport.

Coupling of Metabolism to Transport

Transport systems and carriers. The basic

principles of nutrient transport in microorganisms were set forth in a classic paper by Cohen and Monod (74). Microbiologists ever since have held it to be virtually self-evident that the plasma membrane is largely impermeable to the majority of polar molecules, including nutrients and metabolites. Uptake, when it occurs, is usually not a matter of "permeability" but of transport; that is, it depends upon interaction of the substrate with a component of the membrane which recognizes the substrate with a high degree of specificity, and translocates it across the membrane in a catalytical manner. The neutral term "transport system" will be used here (in preference to "permease") to designate in an operational sense entities that are recognized on the basis of genetic, kinetic, or other criteria to be involved in the translocation of a substrate across the membrane.

Kinetic and genetic analysis of many microbial transport systems (reviews: 74, 186, 206, 207, 242, 292) gave rise to the belief that the substrate combines reversibly with a "carrier"; the resulting complex is often said to be "mobile in" or to "shuttle across" the osmotic barrier. Molecules possessing the attributes of carriers in this sense are known: hemoglobin in saline solution, for instance, or valinomycin in a lipid phase. But microbial transport systems appear to involve protein molecules firmly embedded in the membrane, such as the M protein of the β-galactoside transport system of *E. coli* (206, 188). The Na^+, K^+, ATPase, of mammalian cell membranes and mitochondrial membrane porters (71) are likewise large protein molecules. Models based on the concept of a carrier molecule freely diffusible in a homogenous liquid are thus likely to be unrealistic. We must probably think in terms of conformational changes in a protein molecule, perhaps quite limited in magnitude, which modify the affinity of a binding site for its substrate and also alter the accessibility of the site from one side of the barrier to the other. (I have found the models in references 178, 185, 186, 206, 242 particularly instructive.) Mitchell (258, 259) has stressed the analogy of the active site of an enzyme to the "carrier center" of a porter and points out the important corollary that kinetic and energetic characteristics of the translocation in one direction need not be identical with those in the opposite: carrier centers do not shuttle, they circulate.

At this point we must digress for a moment to consider the relationship of these largely hypothetical carriers to the concrete and ever more numerous "binding proteins." (For recent reviews see references 186, 194, 242, 292, 301.) Proteins which bind specific amino acids, sugars, vitamins, and ions with high affinity are easily released from gram-negative bacteria by osmotic shock or by conversion to spheroplasts. Genetic studies (12, 37, 146, 194, 236, 290) provide persuasive evidence that these binding proteins are essential components of transport systems, particularly of those with high affinity. Reports of changes in conformation and in binding constants (36, 194, 292) lend weight to the hypothesis that binders recognize the substrate at the external cell surface. There is, however, little to suggest that the binders serve to translocate substrates across the plasma membrane: that may be the province of far more hydrophobic molecules such as the M protein, which remain embedded in the membrane. The persistence of specific transport systems for a wide variety of sugars and amino acids in membrane vesicles of *E. coli* (188, 208) and other organisms argues against obligatory involvement of the periplasmic binding proteins in the translocation step. Finally, dissociable binding proteins are not found in gram-positive bacteria, whose protoplasts apparently retain normal transport capacities. Perhaps the function of binding proteins is connected with the complex, multilayered envelope of gram-negative bacteria (but see reference 292).

A striking feature of bacterial transport systems is their capacity to achieve large apparent concentration gradients; 1,000:1 is not uncommon, and K^+ can be transported by *E. coli* to a concentration gradient of 10^6:1. It is often difficult to attach a precise meaning to the concentration of a metabolite in the cytoplasm, and small pools may well exist in a bound state. But the large pools of sugars, amino acids, and other metabolites, which may attain 0.1 to 0.2 M in the cytoplasm, simply exceeded reasonable concentrations of any possible macromolecular receptors. Moreover, many experiments suggest that such pools are osmotically active and hence in "free" solution (2, 3, 41, 45, 72, 96, 163, 185, 188, 219, 308, 347, 401; but also 77 for an alternative view). Such concentrative uptake implies the performance of work by the cells and interaction of the carrier centers with the metabolic machinery.

Group translocation. Group translocations are processes in which passage of the substrate across a membrane occurs concomitantly with, and as a consequence of, chemical transformation of the substrate. It is thus a chemical group, rather than an intact molecule, which

traverses the barrier (259, 321). Thirty years ago it was thought that uptake of sugars by the mammalian intestine may be linked to phosphorylation, but after this was shown to be untrue the concept of group translocation fell into disfavor. It is one of the lesser ironies of history that today the transport system most fully understood is precisely the vectorial phosphorylation of sugars.

(i) *Transport of sugars by vectorial phosphorylation.* In 1964, Kundig and Roseman discovered the existence of a novel system for the phosphorylation of sugars in bacterial extracts. The sequence of reactions shown below is now generally held to account for both the transport of sugars and the initial step in sugar dissimilation by many bacteria. The system is widely, but not universally, distributed (319).

$$(1)\ \text{PEP} + \text{HPr} \underset{}{\overset{\text{I, Mg}^{++}}{\rightleftharpoons}} \text{HPr} \sim \text{P} + \text{pyruvate}$$

$$(2)\ \text{HPr} \sim \text{P} + \text{sugar} \xrightarrow{\text{II, III}} \text{sugar}' - \text{P} + \text{HPr}$$

$$\text{Sum: PEP} + \text{sugar} \xrightarrow[\text{HPr, Mg}^{++}]{\text{I, II, III}} \text{sugar} - \text{P} + \text{pyruvate}$$

The extensive work on this system has been repeatedly and lucidly reviewed (185, 186, 242, 321), so that a summary of the main points will suffice here. The first reaction is the phosphorylation of a small protein, designated HPr, by phosphoenolpyruvate (PEP); the phosphoryl group is linked to a histidine residue. This reaction is catalyzed by enzyme I which, like HPr, is constitutive and soluble. The second step, which is much more complex, consists of the transfer of the phosphoryl group to any of a number of sugars. This reaction is catalyzed by a family of membrane-bound enzymes, some constitutive and some inducible, having varying degrees of specificity. Among the sugars metabolized by the phosphotransferase system of *E. coli* are glucose (and its analogue, α-methylglucoside), mannose, fructose, mannitol, and β-glucosides; a specific enzyme II appears to exist for each. Recently Kundig and Roseman (228, 229) described the further resolution of enzyme II into enzymes IIA (glucose, mannose, and fructose each required a particular IIA protein), and enzyme IIB (common to all three). In addition, the system requires phosphatidylglycerol for optimal activity (228, 253). In *Staphylococcus aureus*, a sugar-specific soluble protein (factor III) is needed in addition to enzymes I and II.

It was clear from the outset that the phosphotransferase system is required for the metabolism of many sugars since mutants deficient in either enzyme I or HPr fail to grow on any of a large number of sugars; mutants that lack a sugar-specific enzyme II fail to grow only on that particular sugar. But it was not at all self-evident that the phosphotransferase system is also required for translocation: it should be recalled that glycerol, for example, passes across the membrane as such and is subsequently phosphorylated by a soluble kinase (see 242 for references). It is therefore necessary to devise experiments to discriminate between two alternative possibilities.

(i) The phosphotransferase system is required only to phosphorylate sugars subsequent to their translocation into the cytoplasm and serves to trap the sugar.

(ii) Phosphorylation occurs concomitantly with, and is required for, the translocation process itself.

The evidence, marshalled in detail in the reviews cited above, overwhelmingly supports the thesis that transport of many sugars by *E. coli* and other bacteria occurs by "vectorial transphosphorylation" (185). Among the principal findings with intact cells is that the sugar first appears in the cytoplasm as a phosphorylated derivative, and the shorter the duration of uptake the greater is the proportion of phosphate ester. In *Staphylococcus aureus*, phosphorylation can be taken to be essential for the uptake of lactose because the β-galactosidase found in the cytoplasm attacks only lactose phosphate, not the free disaccharide. In mutants unable to phosphorylate the sugar, equilibrium is attained very slowly. Similarly, in mutants of *Salmonella* deficient in enzyme I, the maximal rate of α-methylglucoside uptake is at least 50-fold less than in the wild type; qualitatively similar findings with other sugars support the contention that enzyme I is required at least for transport at the normal rate (slow translocation of the sugars by diffusion or by other transport systems does occur). The argument is greatly strengthened by studies on membrane vesicles (see 185–187) which lack cytoplasmic enzymes; under appropriate conditions, all the glucose of α-methylglucoside accumulated by vesicles is found as the phosphorylated derivative in the lumen. Vesicles prepared from mutant cells deficient in enzyme I neither phosphorylated nor transported it. In an ingenious double-label experiment, Kaback (184) found that internal glucose-6-phosphate was almost entirely derived from ^{3}H-glucose added externally; ^{14}C-glucose already present in the vesicles was not phosphorylated. If all this falls short of absolute proof, it does establish beyond reasonable

doubt that translocation of glucose and several other sugars occurs by vectorial phosphorylation.

The focus of interest is now shifting to the molecular events, particularly the precise relationship of phosphorylation to translocation. Several recent findings pertain to this. In *Staphylococcus*, HPr ~ P phosphorylates factor III, and it is this latter protein which is the immediate phosphoryl donor to the sugar (282). In *E. coli*, however, Rose and Fox (320) have obtained preliminary evidence that enzyme II is phosphorylated and in turn donates the phosphoryl group to the sugar. The implication is that the substrate-specific enzymes of the kind designated IIA both phosphorylate and translocate the substrate. The balance of available evidence suggests that translocation can occur only in conjunction with phosphorylation (184, 326), but it is perhaps not entirely excluded that enzyme II, in the absence of phosphorylation, can mediate slow equilibration or at least exchange of their substrates across the membrane. Another question that remains unsettled is the possible existence of an additional sugar-specific element, distinct from enzyme II, whose role is to facilitate access of the substrate to the site of phosphorylation. Thus, enzyme II would phosphorylate its particular sugar but not translocate it. This possibility has been suggested repeatedly, most explicitly so by Gachelin (120), whose argument again turns on the ability of mutants defective in enzymes I or II to carry out at least facilitated diffusion across the membrane. There is at this time no genetic evidence for the existence of a sugar-specific translocation.

Since the phosphotransferase system is unquestionably the main vehicle for transport and dissimilation of some sugars, could it be a universal mechanism? Apparently the answer is no. In *E. coli*, the β-galactoside transport system can be differentiated from the phosphotransferase complex by two experimental observations. Accumulation of β-galactosides is blocked by uncouplers, whereas that of α-methylglucoside is enhanced—perhaps because the uncouplers stimulate production of phosphoenolpyruvate (reviewed in 186, 242). Moreover, as Barnes and Kaback (24) demonstrated, uptake of β-galactosides by membrane vesicles of *E. coli* can be coupled directly to respiration, under conditions in which participation of phosphoenolpyruvate can be virtually excluded (e.g., in a mutant deficient in enzyme I of the phosphotransferase). The suggestion that the phosphotransferase may be the catalyst of galactoside transport has recently been revived by Koch (221), but for the present the weight of the evidence favors transport by two fundamentally distinct mechanisms.

(ii) *Possible uptake of purines, pyrimidines, and fatty acids by group translocation.* The principle of group translocation is compatible with a wide range of metabolic transformations, of which phosphorylation is but the most familiar one. Whenever a substrate appears on the inside rapidly and predominantly in the form of a derivative, group translocation may be suspected. There is probably no need to caution against facile application of what is only a rule of thumb.

For some time evidence has been accumulating that the uptake of purines and pyrimidines by bacteria is intimately related to their utilization. Especially when supplied at low extracellular concentrations, these are incorporated into nucleic acids without any lag and removed from the medium at a rate controlled by the rate of nucleic acid synthesis (53, 284). In *Bacillus subtilis*, Berlin and Stadtman (28) found that 95% of the adenine taken up by resting cells was present as adenosine monophosphate (AMP). The specific activity of the AMP pool was higher than that of internal adenine itself. Such findings may indicate compartmentation but are also compatible with the proposal that uptake of adenine occurs concurrently with its conversion to AMP. Thymine uptake by *E. coli* likewise appears to be closely related to its conversion to deoxythymidine phosphate (195, 196). It is noteworthy that many enzymes catalyzing metabolism of nucleosides and bases are readily released by osmotic shock and may be localized in the periplasmic space.

A thorough investigation of purine uptake by *E. coli* has now been reported by Hochstadt-Ozer and Stadtman (159–161). Again, virtually all the adenine taken up was transferred to AMP and other nucleosides, apparently by the action of purine phosphoribosyltransferase (adenine + PRPP $\rightleftharpoons$ AMP + pyrophosphate). The initial rates of adenine uptake were closely correlated with the amount of this enzyme present, which appears to be localized either in the membrane or else in the periplasmic space. In cells depleted of energy reserves, exogenous phosphoribosyl pyrophosphate (PRPP) greatly stimulated the uptake of adenine; since the kinetic parameters were similar to those found for the isolated enzyme, the conversion of adenine to AMP appears to involve a site accessible to both adenine and PRPP from outside the osmotic barrier (161).

Even though most of the phosphoribosyltransferase was lost from the cells upon osmotic shock, a fraction was retained when membrane vesicles were made. Uptake of adenine by the vesicles was again greatly stimulated by PRPP and resulted in conversion of the adenine to AMP which appeared inside the vesicles (160). It is thus likely that uptake of adenine occurs by agency of the transphosphorylase and represents a novel kind of group translocation. However, the apparent involvement of PRPP from the external, rather than the internal, surface of the membrane is a puzzling aspect of the proposed mechanism.

Uptake of fatty acids by *E. coli* is closely coupled to their degradation: free fatty acids do not appear in the cytoplasm, and mutants genetically defective in fatty acid catabolism have correspondingly reduced rates of uptake. In particular, mutants lacking acyl coenzyme A synthetase do not take up fatty acids. Klein et al. (212) therefore suggest that fatty acid uptake represents yet another case of group translocation, by "vectorial acylation." Whether there exists another element which facilitates access of the fatty acid substrate to the acyl synthetase is uncertain. In this connection it may be mentioned that thiamine uptake by *E. coli* is closely coupled to its phosphorylation by an ATP-dependent kinase associated with the membrane. Here, however, studies with mutants suggest that it is thiamine itself which may be translocated; the kinase is not thought to be required for translocation, only for phosphorylation (199, 200). The distinction between group translocation and translocation followed by chemical transformation is not always easily made.

(iii) *Vectorial biosynthesis of cell envelope polymers.* Peptidoglycan, lipopolysaccharides, and teichoic acids of the cell envelope are localized outside the osmotic barrier. Since precursors for their biosynthesis are produced in the cytoplasm, some mechanism must exist for the transport of building blocks from the cytoplasm to the site of their assembly into polymers. The discovery that a C_{55} polyisoprenoid alcohol serves as glycosyl carrier in peptidoglycan synthesis proved to be of seminal importance: Not only are such compounds involved in the biosynthesis of envelope polymers generally, but they remain the only examples of natural transport carriers of chemically defined structure. The chemical structures of the polyisoprenoid alcohols and their role in the biosynthesis of peptidoglycan, teichoic acids, capsular polysaccharides, lipopolysaccharides, and other polymers have been comprehensively reviewed (93, 323) and will not be further considered here. The biosynthesis of the cytoplasmic membrane itself also falls beyond the scope of this article.

Kinetic approach to energy coupling. Britten's warning (45) that "the transport of small molecules into and within the bacterial cell does not now appear to be an elementary process" is still timely. What insight has been achieved stems primarily from the exhaustive analysis of the β-galactoside "permease" of *E. coli* and inevitably hypotheses formulated from the study of this system have come to dominate our thinking on microbial transport in general. It is neither possible nor necessary to reiterate here material thoroughly covered in the reviews of Kennedy (206), Lin (242), and Kepes (207), except to set forth three conclusions which bear directly upon the coupling of transport to energy-generating metabolism. (a) Exit of galactosides from *E. coli* is, like entry, a carrier-mediated process. Both genetic studies and experiments with inhibitors suggest that the same carrier center mediates both entry and efflux (though it should be kept in mind that more than one pathway may exist for each). (b) There is no evidence that phosphorylation, acetylation, or any other chemical modification of β-galactosides occurs during translocation. With the exception of a recent paper by Koch (221), current models agree that coupling of transport to metabolism occurs at the level of the carrier rather than that of the substrate. (c) Recognition of the β-galactosides and its translocation *per se* do not require metabolic energy; coupling to metabolism is necessary only for accumulation against a concentration gradient.

The hypothesis that β-galactoside transport occurs by facilitated diffusion with facultative energy coupling was first stated by Koch (220) and subsequently confirmed under a variety of conditions (58, 198, 303, 399). It rests primarily on experiments with metabolic inhibitors, especially proton-conducting uncouplers (azide, dinitrophenol, CCCP). These stopped accumulation of thiomethylgalactoside (TMG) and other nonmetabolizable substrates of the transport system and caused rapid efflux of any TMG previously accumulated by the cells. They did not, however, inhibit translocation *per se:* hydrolysis of *o*-nitrophenyl galactoside (ONPG) was only slightly inhibited and both counterflow and equilibration of the substrate across the membrane were still observed. Winkler and Wilson (399) measured the apparent dissociation constants, K_m, of the influx and efflux steps; in metabolizing cells, the

K_m for efflux was lower by a factor of 25 than that for influx, but in presence of an uncoupler they were the same. It was proposed that energy coupling, whatever its nature may be, occurs on the inner surface of the membrane and lowers the affinity of the carrier for its substrate which consequently accumulates in the cytoplasm. Uncouplers interfere with energy coupling and thus equalize the affinities at the two surfaces. Wong et al. (400) recently confirmed this conclusion by detailed kinetic analysis of the effect of various inhibitors on the uptake of TMG and ONPG.

Strong support for the view that energy coupling converts a facilitated-diffusion system into one capable of active transport comes from the isolation of a mutant deficient in the energy-coupling step (397, 402). This mutant had largely lost the ability to accumulate β-galactosides despite somewhat enhanced capacity for carrier-mediated translocation. The mutation maps at the far end of the Y cistron (T. H. Wilson, *personal communication*).

Finally, Kashket and Wilson (198) showed that *Streptococcus lactis* can carry out facilitated diffusion of TMG even in the absence of an exogenous energy source; addition of glucose supports concentrative transport of TMG and other galactosides.

These observations, and the very large body of kinetic measurements not mentioned here, are accounted for by models of the type illustrated in Fig. 12 (206, 207, 399). This particular model has been analyzed in detail by Kepes (207). Briefly, the permease or carrier (identical with Kennedy's M protein) both recognizes and translocates the substrate. Pathways *abcg* and the reverse route *cba* require no metabolic coupling and account for equilibration, counterflow, and exchange of

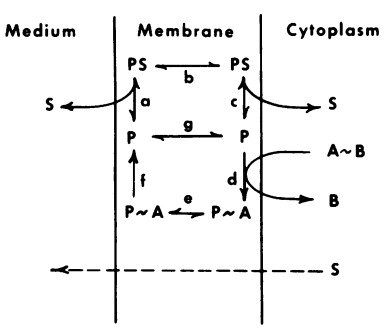

FIG. 12. *Kinetic model of β-galactoside transport. After Kepes (207). P, "Permease" or carrier center; A ~ B, hypothetical energy donor; P ~ A, energized from of permease; S, substrate; dashed line, various unspecified exit processes.*

galactosides in the presence of inhibitors. When cellular energy metabolism is intact, the carrier can undergo a coupling reaction, pathway *d*, which lowers its affinity for galactosides. The original, high-affinity state of the carrier is restored by the energy-dissipating sequence *ef*.

The view that energy is required *only* for accumulation but not for translocation *per se* is not universally accepted. Manno and Schachter (244), for example, interpreted their kinetic studies as indicating an energy-requiring entry process; the validity of their methods has, however, been questioned by Kepes (207). The most serious challenge comes from Koch (221), whose earlier work has led to the formulation of energy coupling as a facultative element of transport. Koch subjected *E. coli* to starvation in the presence of α-methylglucoside, in order to make the cells expend metabolic energy on a useless transport cycle and thus deplete their reserves. Such "exercised" cells no longer hydrolyzed ONPG, even though they still possess both β-galactosidase and the transport system. Addition of minute amounts of glucose permitted rapid but limited hydrolysis of ONPG, with an approximate stoichiometry of 25 to 30 moles of ONPG per mole of glucose. Koch (221) concluded that influx of ONPG, even downhill, inherently requires expenditure of energy, and proposed a model in which galactosides are phosphorylated via the phosphotransferase system, followed by hydrolysis via a phosphatase. Presumably, stoichiometric modification of the carrier, rather than of the substrate, could equally well account for his observations. I am not persuaded that Koch's results *require* us to discard the conventional view supported by evidence of many different kinds, that accumulation depends upon a manner of coupling to cellular metabolism which is not necessary for translocation as such. They may, however, suggest the need for at least minimal metabolism to keep the transport systems in a functional state.

Even more debatable than the kinetic consequences of energy coupling are the nature of the energy donor and of the energy-coupling reaction (*d*). Kepes favors the postulate that "energy coupling involves a covalent reaction with the energy donor and permease protein (or a complex including permease protein). It is believed that the result of the reaction is the establishment of a covalent link between permease and one of the radicals of the energy donor according to the example of the phosphorylation of the Na^+-K^+-dependent ATPase.

The derivative, symbolized P ~ A, will be called the energized form of permease" (207). Uncouplers might either prevent formation of the donor A ~ B or act at one of the subsequent steps.

Could the energy donor, A ~ B, be ATP? ATP does enhance translocation of β-galactosides under certain conditions (329, 387). However, two lines of evidence suggest that the role of ATP is an indirect one: (i) *E. coli* accumulates TMG even under anaerobic conditions; accumulation is inhibited by proton-conducting uncouplers which do not significantly affect the ATP pool (303). (ii) More compelling is the finding that membrane vesicles of *E. coli* can couple respiration directly to the accumulation of galactosides (24). Thus far, the covalent energy donor A ~ B remains hypothetical and quite as elusive as the chemical intermediates of oxidative phosphorylation.

Kepes (207) leaves open the possibility that A ~ B may, in fact, be a high-energy intermediate of oxidative phosphorylation, but there is a plethora of attractive alternatives. Kaback, whose work will be discussed in the following section, suggested that the β-galactoside carrier may itself be a member of a redox chain, and suffers cyclic changes of orientation and affinity by oxidation and reduction of critical sulfhydryl groups. But there is no compelling reason to assume any covalent modification of the carrier center at all. Lowered affinity of the carrier at the interior surface, or lowered mobility, could be due to an appropriate gradient of ion activity across the membrane (*see below*). Finally, Boyer and Klein (43) have developed a model in which energy input brings about a conformational change in membrane structure, such as to block access of substrate from the inside to the carrier; this model does not invoke a lowered affinity of the carrier for its substrate at all. Speculation is not seriously hampered by facts, and transport workers know for their sins that it is easier to construct models than to subject them to critical test.

How broadly applicable are generalizations drawn from β-galactoside transport—and especially the hypothesis that accumulation against a concentration gradient requires coupling to metabolism, but translocation *per se* does not? For many transport systems, little more is known than that dinitrophenol and other inhibitors block accumulation and, in some cases, discharge pools previously accumulated. Examples from *E. coli* include galactose (167), arabinose (289), sulfate (87), phosphate (249), leucine (305), aromatic amino acids (50, 51), magnesium, and manganese (243, 345, 346). Citrate transport in *B. subtilis* (392), α-aminoisobutyrate in *Bacillus megaterium* (245), and proline uptake by *Pseudomonas* (201) are similarly inhibited. Rather more informative is the finding that, in *E. coli*, exchange of amino acids between the medium and the internal pool continues under conditions which prevent net accumulation of amino acids (46, 209). Similar observations were made in *Pseudomonas:* an uncoupler, azide, inhibits accumulation of proline but not its entry into the cells (201). Recently, Hechtman and Scriver (147) described a mutant which can transport β-alanine across the membrane but does not accumulate it against a concentration gradient, and suggested that the genetic lesion is in the energy-coupling step. Fragmentary as it is, the evidence does suggest that in the case of amino acids, translocation and accumulation have different requirements for metabolic energy.

Streptococcus faecalis has many virtues, among which the apparent absence of internal energy reserves and of oxidative phosphorylation are particularly pertinent to attempts to explore the nature of energy coupling. Several transport systems in this organism appear to be strictly dependent on an external energy source, including those for sucrose (3), glycylglycine (2, 3), phosphate (133), potassium (134), and glutamate (314). Some amino acids, however, can enter the cells by exchange for other members of the cellular amino acid pool even in the absence of glucose: glycine, threonine, alanine, serine, and their analogue, 2-amino-3-phosphonopropionic acid, fall into this category (47, 164, 275). Glucose stimulates accumulation of the latter amino acids; glucose-stimulated uptake, unlike the exchange, is inhibited by proton-conducting uncouplers, yet both appear to be mediated by the same transport system (136; Asghar and Harold, *unpublished data*). For these amino acids, then, translocation by exchange does not require an energy source, but net accumulation beyond this point does. The same conclusion was reached by Kashket and Wilson (198) for TMG uptake by *S. lactis*.

Coupling of transport to the respiratory chain in membrane vesicles. A milestone in the continuing analysis of bacterial membrane function was passed with the description, by Kaback and Stadtman (192), of a membrane preparation from *E. coli* which carried out active transport of proline. I propose to review the results of this trial of research in some de-

tail, both because of its importance as a technique of broad applicability and because it has led to iconoclastic views that deserve critical scrutiny.

Vesicles are prepared by osmotic lysis of spheroplasts under carefully specified conditions (184, 185, 187); they are usually closed sacs of relatively uniform size bounded by a single unit membrane, but some strains yield multi-layered structures. Much effort was expended on the difficult task of proving that the vesicles are topologically closed and possess the same orientation as the parent cells, i.e., right-side out (185, 188, 190). The vesicles are practically devoid of cytoplasmic enzymes and cofactors. They also carry out no detectable ATP synthesis by oxidative phosphorylation; whether this is due to loss of essential coupling factors or simply reflects the orientation of the intact vesicles remains to be determined. However, when provided with a suitable energy source, the vesicles accumulate a variety of substrates against the concentration gradient and at a rate at least comparable to that of the parent cells: β-galactosides (24, 25, 189), galactose, and other sugars (208), a variety of amino acids (190, 191, 192), manganese (29), and, in presence of valinomycin only, K^+ and Rb^+ (30, 188).

Accumulation of these substrates requires presence in the membrane of the appropriate transport systems. Thus, vesicles derived from mutants deficient in transport of β-galactosides, proline, or glycine share the deficiency of the parent cells. However, vesicles appear to be devoid of binding proteins, which are easily lost by osmotic shock. The binding proteins thus appear to play no indispensable role in translocation across the plasma membrane; however, the transport of galactose by vesicles occurred with relatively low affinity, suggesting that the binding proteins are involved in conferring high affinity upon certain transport systems (208). Accumulation also depends upon the integrity of the barrier functions of the membrane, and thus upon membrane lipids: vesicles can be rendered leaky and resealed by various manipulations, including changes in temperature (185, 188, 189). Unlike the uptake of glucose, which occurs by group translocation, there is no evidence for any chemical transformation of the substrates during concentrative uptake driven by respiration. Moreover, the accumulated substances appeared to be in free solution in the lumen of the vesicles. Internal concentrations may be of the order of 15 mM, with concentration gradients of 100:1 (189).

Most of the available information comes from vesicles of *E. coli* membranes. However, preparations capable of concentrative transport have also been obtained from *S. aureus* (344), *B. subtilis* (222, 223), *Mycobacterium phlei* (157), and other organisms (222). Membrane vesicles of *S. faecalis* were unable to couple respiration to active transport, in accordance with the general belief that this organism normally relies entirely upon substrate-level phosphorylations for metabolic energy.

Accumulation of substrates is almost completely dependent upon respiration, and it is the nature of the coupling between these processes that has been the focus of recent papers by Kaback and his associates. (The published information refers predominantly to the uptake of β-galactosides, but qualitatively similar findings were made with other metabolites [188, 208, 344]). The cornerstone of the argument is the contention that ATP, derived from oxidative phosphorylation, is *not* an intermediate in the coupling of respiration to transport. The case rests partly upon the absence of detectable ATP from the vesicles, which also did not respond to ATP added externally. More convincing is the finding that even high concentrations of arsenate, which is known to uncouple oxidative phosphorylation in intact cells, did not inhibit respiration-linked transport by vesicles (24, 43, 157, 208, 213). Moreover, DCCD, a standard inhibitor of ATPase, did not interfere with uptake of proline by vesicles of *M. phlei* (157). The linkage between respiration and transport thus appears to be a fairly direct one.

By no means do all substances oxidized by the vesicle preparations also support transport. Vesicles of *E. coli*, for example, oxidize NADH, D-lactate, and succinate, in that order of preference. However, D-lactate was the most effective substrate for transport, followed by succinate, formate, α-glycerol phosphate, and L-lactate; NADH was a poor energy donor (25). Subsequently, the combination of ascorbate and phenazinemethosulfate was found to be an excellent energy donor for β-galactoside transport by vesicles of *E. coli* (and of amino acids by vesicles from other sources [157, 222, 223]). In all cases, oxidation was mediated by an electron transport chain. Since D-lactate, succinate, and NADH all reduced the cytochrome components of the membrane, and to the same extent, it appears that reducing equivalents from all the substrates traverse the same common cytochrome chain (Fig. 10). D-Lactate was the best energy donor for transport, and therefore Barnes and Kaback (25) placed the

site of energy coupling of respiration to transport between D-lactate dehydrogenase and cytochrome *b*. Kerwar et al. (208) reached the same conclusion with respect to galactose uptake.

The localization of the coupling site was supported by the application of inhibitors. Sulfhydryl reagents, especially *p*-chloromercuribenzoate and *N*-ethyl maleimide, inhibited both lactate oxidation and transport; both inhibitors were reversed by dithiothreitol. D-Lactate dehydrogenase, when assayed separately, was completely indifferent to sulfhydryl inhibitors. Therefore the coupling site must again lie subsequent to the dehydrogenase, and a close relationship was inferred between oxidation and transport (189). By contrast, oxidation of NADH was not sensitive to sulfhydryl reagents and appears to involve a separate chain as far as cytochrome *b*.

Other inhibitors of respiration also block both oxidation of D-lactate and accumulation of β-galactosides. Of key importance to the argument is the observation that anaerobiosis, KCN, and hydroxyquinoline *N*-oxide (HOQNO) stop accumulation and also induce efflux of galactosides previously accumulated by the vesicles. By contrast, *p*-chloromercuribenzoate, and also oxamate (which inhibits D-lactate dehydrogenase itself) were shown to block accumulation *without* inducing efflux (189).

These observations are the main pillars supporting the tentative model put forth by Kaback and Barnes (189). They suggest (Fig. 13) that a specialized electron transport chain connects D-lactate with cytochrome *b*; NADH has little or no access to this chain. The transport carrier for β-galactosides, and indeed every transport carrier, is either a member of this special oxidation chain or else closely linked to its redox carriers. The transport carrier is proposed to exist in two states, differing in orientation and in affinity for the substrate. The oxidized form, S-S, has high affinity for β-galactosides at the external surface. Upon reduction of the disulfide bond, a reorientation occurs; the substrate is translocated to the inner surface and concomitantly the affinity of the carrier center is lowered. Thus the substrate dissociates from the carrier and accumulates in the lumen of the vesicle. It is furthermore suggested that the reduced, low-affinity form of the carrier can mediate efflux of the substrate across the membrane, as well as exchange. The latter postulate is needed to explain the remarkable observation that inhibitors which act beyond cytochrome *b* not only inhibit accumulation but also induce efflux of any galactoside previously accumulated: The electron transport chain, including the carrier, would be expected to be reduced under these conditions. Conversely, oxamate for example, inhibits accumulation but does not elicit efflux; according to the model, the carrier remains in the oxidized (high-affinity) state in the presence of inhibitors that act before the coupling site.

It seems to me quite solidly established that the vesicles couple respiratory energy to transport by a mechanism which does not involve ATP. What is at issue is the precise nature of the coupling, and it is my opinion (*pace* Kaback) that some of the data now available admit of alternative interpretations.

The first doubts arise from topological considerations. Right-side-out vesicles would not be expected to oxidize NADH nor to hydrolyze

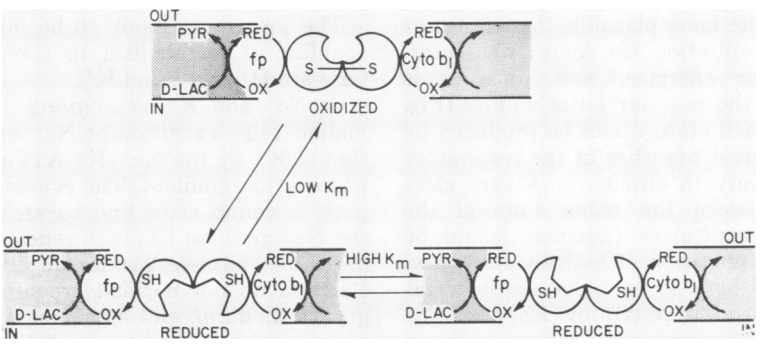

FIG. 13. *Coupling of transport to D-lactic acid dehydrogenase, according to Kaback and Barnes (189). D-LAC, D-lactate; PYR, pyruvate; fp, flavoprotein; Cyto b_1, cytochrome b_1; OX, oxidized; RED, reduced. OUT signifies the outside surface of the membrane; IN signifies the inside surface. The hemisphere located between fp and cyto b_1 represents the "carrier": ω, a high-affinity binding site; and W, a low-affinity binding site. The remainder of cytochrome chain from cytochrome b_1 to oxygen has been omitted.*

ATP, since the requisite enzymes are almost certainly localized at the inner surface. Thus the reported synthesis of phospholipids from ATP (385), ATPase activity, and NADH oxidase may betray a fraction of vesicles which are either open or, more likely, damaged in some more subtle way. It is at least arguable that the failure of NADH or of ATP to support transport is due simply to their inability to reach their proper site of action at the inner surface of "competent" vesicles. If this be true, we would have to attribute the efficacy of D-lactate, succinate, and glycerol phosphate to the retention by the vesicles of transport systems mediating their entry. Furthermore, although D-lactate is the best energy source, substrates of other oxidation pathways do also serve: succinate, L-lactate, glycerolphosphate, and formate were half as effective as D-lactate in supporting the initial role of lactose uptake and supported about one-third of the maximal pool size (25). Kaback (*personal communication*) suggests that these substrates are oxidized partly via the lactate dehydrogenase chain, partly by other routes—an *ad hoc* hypothesis which seems needlessly complex. Incidentally, the fact that many bacteria, including *E. coli*, can grow anaerobically at the expense of glycolysis is an *a priori* argument against redox intermediates as transport carriers.

The most glaring deficiency of the model of Kaback and Barnes (189) is its failure to account explicitly for the striking inhibition of transport by uncouplers and by valinomycin in presence of K^+ (24, 25, 157, 208). The very fact that these compounds do not inhibit respiration, but only dissociate it from transport, implies that cyclic oxidation and reduction of electron carriers is not by itself sufficient to drive active transport.

It seems to me more plausible that transport is linked to respiration via energy-rich intermediates or an energized state, just as is thought to be the case for mitochondria (Fig. 1). This energized state would be produced by any one of several branches of the respiratory chain, which vary in efficiency. A very close correlation between the redox state of the chain and concentrative transport would be expected, but inhibition of both by sulfhydryl reagents would have to be explained in terms other than those suggested by Kaback. The nature of the energized state and the manner in which it drives concentrative transport is a separate issue: Hypotheses can be framed in terms of chemical, conformational, or chemiosmotic coupling (e.g., 43, 189, 265, 361). But I would stress that, as was earlier found to be the case for mitochondria, it is necessary to account for the dissipation of the energized state by reagents which conduct protons or K^+. A relationship between transport by cells or vesicles and ion gradients has by no means been "ruled out" (25, 188): on the contrary, there seems to be quite a lot to be said for it.

Ion gradients and energy coupling. The hypothesis that ion gradients are the link between transport and metabolism rests upon the presupposition that certain metabolic pathways catalyze reactions oriented across the membrane, and thus generate primary gradients of metabolites. Because they bear an electrical charge, gradients of ions are more likely to serve in energy coupling than are gradients of neutral molecules. The primary ion gradients support the transport of other nutrients or metabolites against the electrochemical gradient by means of a hierarchy of secondary carriers, which have affinity sites for both the coupling ion and for particular nutrients. In the case of symport, the substrate and the ion are translocated in the same direction; the driving force on the coupling ion, which may have an electrical component as well as a concentration component, is exerted also on the substrate passenger and thus brings about "active" transport of the latter. Antiport designates the reverse situation, in which the coupling ion moves in one direction, the passenger in the other, so that a previously established ion gradient can set up an opposed gradient of passenger. These mechanisms of "energy coupling" are analogous to the more familiar situation in which the product of one enzymatic reaction is a substrate of a second: here it is the driving force of one translocation that is balanced against a second translocation (256, 264, 265).

The ions most likely to be involved in the coupling of metabolism to transport in bacteria are H^+, Na^+, and K^+.

(i) *Na^+ and K^+ as coupling ions.* In mammalian cells, extrusion of Na^+ and accumulation of K^+ by the Na^+,K^+-ATPase provides a primary ion gradient. The evidence that transport of amino acids and sugars is coupled to the Na^+ gradient has been repeatedly reviewed (341, 358; but see 211 for an opposing view). Bacteria do not normally require Na^+ in the growth medium, and a general role for Na^+ in coupling transport to metabolism seems unlikely. Nonetheless, there are a number of reports of Na^+-dependent transport processes: Na^+ stimulates glutamate transport in *E. coli* (117) and is clearly involved in amino acid

transport by a halophilic *Pseudomonas* (364, 401). Na^+ specifically stimulates uptake of TMG by "permease II" of *Salmonella*; conversely, TMG stimulates ^{22}Na uptake, suggesting symport of the cation and the sugar (359). These intriguing reports suggest that Na^+ may, after all, serve as coupling ion for particular translocation processes, but at this time there is no instance in which a Na^+ *gradient* has been directly implicated as a source of energy for any bacterial transport process.

All bacteria accumulate K^+, and it is *a priori* plausible that they might utilize some of the potential energy stored in this gradient to drive the accumulation of other metabolites. There is at present no evidence in support of this suggestion. Indeed, the finding that many bacteria grow, and presumably transport perfectly well at external K^+ levels close to the internal one, argues against participation of the K^+ gradient in concentrative transport. On the other hand, many investigators have noted an apparent K^+ requirement for various transport processes (2, 3, 133, 135, 275, 295, 329, 401). The function of K^+ is not known. In one cursory study, cells of *S. faecalis* whose K^+ complement had been almost completely replaced by Na^+ were found to be still capable of amino acid and phosphate transport (135), so that K^+ is not obligatory in this system: K^+ may be required only because of its role in the extrusion of protons from the cells (140, 141). In yeast, however, evidence has been adduced by Eddy and his associates (88–90) that movements of K^+ and of Na^+ accompany the uptake of amino acids and that cation gradients supply at least part of the driving force. The possible involvement of K^+ and Na^+ in energy-linked transport by bacteria deserves much more rigorous investigation than it has received so far.

(ii) *Protons as coupling ions.* The hypothesis that the linkage between transport and metabolism is effected by a gradient of proton activity is historically rooted in the familiar observation that uncouplers of oxidative phosphorylation block so very many transport processes. It was the discovery that 2,4-dinitrophenol inhibits uptake of galactose and galactosides by *E. coli* and discharges preexisting pools that led Mitchell (256) to formulate these transport systems as H^+-sugar symport, and to attribute the effect of uncouplers to their ability to facilitate diffusion of protons across lipid membranes.

Conventional wisdom, of course, offers quite another explanation for the inhibition of active transport by uncouplers: Uncouplers interfere with the generation of ATP by oxidative phosphorylation and the inhibition of transport follows secondarily from lack of ATP. Since this syllogism is so often taken for granted, it is desirable to restate explicitly the evidence that refutes it. (i) It has been found repeatedly (38, 39, 225, 316, 398) that uncouplers inhibit transport even under anaerobic conditions. In *S. faecalis* and in anaerobically grown *E. coli*, both of which generate metabolic energy by glycolysis, a series of uncouplers was found to block transport of K^+, phosphate, sucrose, several amino acids, and TMG, even though uncouplers inhibited neither the generation nor the utilization of glycolytic ATP (136, 198, 303). (ii) Membrane vesicles couple respiration to active transport by a mechanism not involving ATP, which is again sensitive to uncouplers (*see above*). Clearly, the effect of uncouplers cannot be attributed to interference with ATP synthesis but must be exerted directly on the membrane.

Uncouplers do, in fact, exert a direct effect on microbial membranes: They facilitate diffusion of protons across the plasma membrane, which is otherwise largely impermeable to them (106, 131, 132, 136, 180, 256, 303, 335). A correlation between the rate of proton movements and the degree of inhibition of phosphate uptake has been reported (136). It is thus a reasonable working hypothesis that the inhibition of active transport by uncouplers may be due to their ability to collapse gradients of *p*H and of electrical potential across the membrane.

In two recent articles, Mitchell (264, 265) has developed in some detail his views on the mechanisms by which, in both mitochondria and bacteria, protons serve to couple metabolism to the uptake of nutrients. As was discussed earlier, the respiratory chain is thought to be arranged across the plasma membrane so as to extrude protons and generate a difference of *p*H and of electrical potential. In anaerobic organisms, proton extrusion may be accomplished by oxidation/reduction of pairs of substrates (e.g., Stickland reaction) via an oxidation chain arranged across the membrane in a loop. Finally the Mg^{2+}-dependent ATPase of bacterial membranes is thought to catalyze the reversible translocation of protons outward when ATP is hydrolyzed and would thus constitute an independent proton pump which can generate the proton gradient at the expense of glycolytic ATP. The asymmetric distribution of protons gives rise to a proton-motive force which, as was discussed previously for mitochondria, is the sum of two compo-

nents reflecting the pH and electrical gradients, respectively. The contributions of ΔpH and $\Delta\Psi$ to the total proton-motive force would vary with the organism and the circumstances.

Aside from a limited number of group translocations, the theory implies that most nutrients are accumulated by secondary porters coupled to metabolism only via the proton-motive force. K^+ accumulation is attributed to an electrogenic porter specific for K^+, and its extent would be a function of the electrical potential. Anion accumulation (e.g., sulfate or phosphate) is seen as anion-proton symport and, if electrically neutral overall, would occur to an extent determined largely by the ΔpH. Finally, accumulation of uncharged metabolites could be mediated by porters that catalyze symport with H^+, as diagrammed for β-galactosides in Fig. 14; association of the porter with protons could determine either its affinity for the ligand or the "mobility" of the complex.

A calculation may render this proposal more concrete. The mechanism as written postulates electrogenic translocation of one proton into the cell with each molecule of β-galactoside. Let us assume, for the sake of illustration, a ΔpH (interior alkaline) of one unit and a $\Delta\Psi$ of -120 mv (interior negative). The total proton-motive force, Δp, would then be -180 mv. An equal force would be exerted on β-galactoside molecules; since a concentration factor of 10 corresponds approximately to 60 mv, galactosides could in principle accumulate in the cells to a concentration gradient of 1,000:1.

In Mitchell's view, then, the metabolism of bacterial cells (like that of mitochondria) is dominated by a circulation of protons between cytoplasm and medium, shown in a greatly simplified form in Fig. 15: Respiration and ATPase translocate protons outward; ATP synthesis and nutrient accumulation are, in general, linked to translocation of protons inward. Now, a steady-state electrical potential across the membrane would cause external cations, K^+ and Na^+, to accumulate in the cells; this would tend to collapse the potential difference and replace it by a rise in the internal pH. To retain a significant potential across the membrane, Mitchell postulates the existence of K^+/H^+ and Na^+/H^+ antiporters whose function is to translocate these cations *outward*. The absolute difference of pH and of electrical potential will thus depend on the rates of proton and other ion translocation, both inward and out. It is a network of multiple interactions, whose complexity will be compounded if Na^+-coupled transport systems prove to be a reality. Quantitative analysis of this web is beyond both the scope of this article and the competence of its author.

Until recently, proponents of the view that active transport is linked in some manner to proton movements had to rest their case largely on the inhibition of transport by uncouplers: not a compelling argument since, as we discussed in the context of mitochondria, the mode of action of uncouplers is still subject to dispute. In particular, one should keep in mind that it may not be possible to distinguish proton conduction across the membrane from conduction to sensitive sites buried in the hydrophobic regions of its interior. During the past few years, however, evidence has begun to appear which points to the generation of ΔpH and $\Delta\Psi$ by bacteria and implicates these in active transport.

In this laboratory, a systematic study was initiated of the relationship of proton movements to nutrient transport in *S. faecalis*—an organism that is convenient precisely because of the absence of oxidative phosphorylation. As far as we have been able to determine, transport is energized entirely by glycolysis and other substrate-level phosphorylations; all the evidence points to ATP as the energy donor

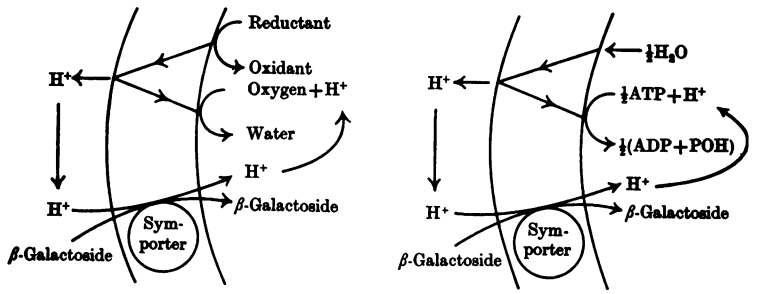

FIG. 14. *Accumulation of β-galactosides in E. coli by a H^+-symport mechanism. From Mitchell (265). β-Galactoside translocation a, when the proton current is due to respiration; b, when the proton current is due to adenosine triphosphate (ATP) hydrolysis.*

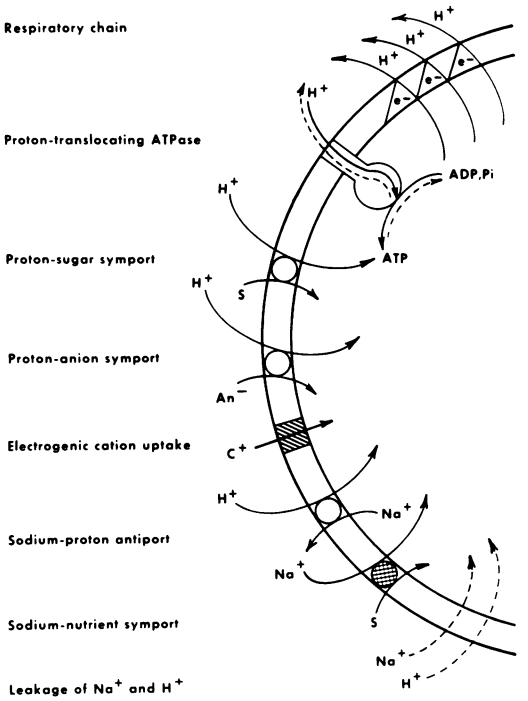

FIG. 15. *Coupling of transport and metabolism by circulation of protons and Na⁺. Proton circulation after Mitchell (265).*

for the accumulation of K^+, phosphate, amino acids, and many other substances. A potent Mg^{2+}-dependent ATPase activity (Table 2) is associated with the plasma membrane. This is a multienzyme complex involving at least three components: the ATPase itself, a protein called nectin that is believed to link the enzyme to the membrane, and an unidentified component of the membrane which confers upon the ATPase sensitivity to DCCD (4, 6, 26, 137, 138, 332, 333). There is little doubt that this enzyme is implicated in the coupling of glycolysis to membrane transport of many nutrients. One exception is the uptake and dissimilation of glucose itself, which apparently involves the phosphotransferase system.

A possible role for the ATPase emerged from studies on the linkage between glycolysis and the accumulation of K^+, in which extrusion of protons from the cells appears to play a central role. Glycolyzing cells generate a *p*H gradient of up to one unit, interior alkaline, by extrusion of protons in exchange for K^+ (139, 142). The *p*H gradient is dissipated by exhaustion of the substrate, by proton-conducting uncouplers, and by antibiotics of the nigericin type, but not by valinomycin (142). More recently, studies on the accumulation of lipid-soluble cations (e.g., DDA^+) provided convincing evidence that extrusion of protons during glycolysis is an electrogenic process which can generate a membrane potential of some -150 to -180 mv (140, 141). The ATPase was clearly implicated in proton extrusion and constitutes part of a proton pump, but its precise role remains to be explored. Accumulation of K^+, quantitatively one of the major transport processes carried out by *S. faecalis*, is ultimately dependent upon the extrusion of protons, since mutations and inhibitors which interfere with proton extrusion also impair K^+ uptake. Indeed, the proton pump is involved also in the expulsion of Na^+ from the cells. There appears to be no Na^+ pump, but rather an electroneutral antiport of Na^+ for H^+; the H^+ is then ejected by the pump (141, 271). As far as they go, these results are consistent with the scheme outlined in Fig. 15. However, unlike the case in mitochondria, the K^+ complement of *S. faecalis* was not discharged by proton conductors and thus does not appear to be in *equilibrium* with an electrical potential across the membrane.

In *S. faecalis*, as in many other bacteria, transport of amino acids, phosphate, and certain sugars is sensitive to proton-conducting uncouplers (136). It is also inhibited by nigericin and by monensin, but not by valinomycin plus K^+. Bearing in mind the known modes of action of these inhibitors (Table 1), it may be that the *p*H gradient may play a role in the transport process, but that a membrane potential does not. Indeed, K^+-replete cells of *S. faecalis* do not now appear to maintain a large steady-state potential (141). Further work is clearly required to clarify the relationship of Δ *p*H and $\Delta\Psi$ to nutrient transport, and especially to search for co-transport of H^+ into the cells together with amino acids and other ligands.

Direct evidence for proton-substrate symport has, however, been presented by West (388): under certain conditions uptake of lactose by *E. coli* is accompanied by stoichiometric influx of protons. More recent results (389) confirm that H^+-lactose symport is an electrogenic process, which would thus be controlled by a membrane potential. In staphylococci, uptake of amino acids may likewise be determined by the membrane potential (286). Eddy and his associates (88–90), using yeast, reported influx of protons together with that of amino acids; they suggested that H^+, and perhaps K^+ also, are co-substrates for amino acid accumulation via an ion gradient.

Do bacterial membrane *vesicles* extrude pro-

tons with the generation of a proton-motive force? This is an issue that is certain to be hotly debated. Reeves (313) has just reported experiments which suggest that membrane vesicles of *E. coli* respiring D-lactate extrude protons into the medium by a process sensitive to uncouplers, and suggested the production of a proton gradient. Subsequent experiments, however, led Kaback (188) to state that the extrusion of protons is observed even under conditions which destroy the permeability barrier. The experimental details will be awaited with interest.

In a number of vesicle preparations, active transport of sugars and amino acids is inhibited by valinomycin plus K^+ (25, 30, 157, 286), which suggests that transport depends in some manner upon an electrical potential, interior negative, that is dissipated by the electrophoretic influx of K^+. It is pertinent here that vesicles of *E. coli*, at least, have apparently lost the transport system for K^+ and take up K^+ only in presence of valinomycin (30). Unlike valinomycin, nigericin did not inhibit the transport of proline by membrane vesicles of *M. phlei* (157); this makes sense since nigericin would not be expected to collapse a potential difference (Table 1). Finally, in the same system (157), transport of proline was uncoupled by lipid-soluble cations but not by anions: further evidence, however indirect, that transport is related to the generation by the vesicles of an electrical potential, interior negative. This conclusion is denied by Kaback (188), who feels that accumulation of Rb^+ by *E. coli* vesicles in presence of valinomycin reflects a pump coupled to the D-lactate dehydrogenase rather than electrophoretic influx in response to the membrane potential. The experimental findings on which Kaback based this view were not available at the time of writing, and further comment would be premature.

There seems to me little doubt that proton translocation is somehow involved in the active transport of many nutrients. Whether the proton circuit (Fig. 15) correctly describes the nature of this relationship remains, however, a matter on which honest men may differ. The concept that metabolism and transport are linked by a proton current depends for its support very heavily on what we believe to be the modes of action of ionophores and other reagents (Table 1). Thus the first requirement is clearly for direct and reliable measurements of membrane potentials, *p*H gradients, and the stoichiometry of proton symport, to put the hypothesis upon firmer footing and to assess its quantitative adequacy. Beyond this, one can discern difficult questions to which answers will have to be sought. For example, the requirement that respiring cells maintain a large electrical potential, interior negative, in the steady state, raises a number of problems. In *S. faecalis*, at least, cells replete with K^+ do not appear to maintain a large potential. Indeed, it is not easy to see how collapse of the potential can be avoided in the presence of high external K^+—such as the 0.1 M buffers frequently used. Mitchell and Moyle (265, 270, 271) postulate the existence of a K^+/H^+ antiporter to extrude such unwanted K^+, but it must be doubted whether such a system, thus far unrecognized, would suffice. There are also various data which suggest that *E. coli* completely devoid of energy sources does not translocate metabolites at all (221). This may be related to the many results from Kaback's laboratory which point to the involvement of sulfhydryl groups in the transport of sugars and amino acids. These and many other findings must eventually find their place in an integrated view of transport, and clearly the time is not yet.

Role of the Membrane in Motility

The role of flagellae in bacterial motility is universally recognized, but the mechanism by which the motion is produced is uncertain. Flagellae may be semirigid helices, activated by a power source located at the base, which imparts to them a rotary motion. Alternatively, the subunits may undergo periodic changes of conformation such that helical waves are propagated from the base of the flagellum to its tip. The fact that removal of the cell wall immobilizes the organisms suggests the need for a rigid structure on which the flagellum exerts its thrust.

No one doubts that motility requires a source of energy, but the identity of the immediate energy donor is not at all established. Sherris and his associates, whose work has been well reviewed by Doetsch and Hageage (86), found that a strain of *Pseudomonas* became immobile when the oxygen supply was exhausted. However, arginine, which is catabolized by pathway that generates ATP by substrate-level phosphorylation, restored motility under anaerobiosis. These results gave rise to the prevailing opinion that flagellae, like muscles, employ ATP as the immediate energy donor. The ATP would be supplied either by oxidative phosphorylation or by substrate-level phosphorylation. However, there is nothing to

exclude the possibility, admittedly speculative, that the plasma membrane is more intimately involved in the generation of motive power. Perhaps the broadest hint in this direction is given by the superb electron micrographs published by De Pamphilis and Adler (84, 85). These reveal many details of the association of flagellae with both wall and membrane, complete with an engineer's view of the hook-up, and provide a structural basis for a role of the membrane in motility more direct than as a source of ATP.

The only other evidence of which I am aware is the finding (102–104) that motility was inhibited by a number of antibiotics which exert their effects upon the membrane. Gramicidin, valinomycin, and monactin, all K^+-conducting ionophores, inhibited motility but neither oxygen uptake nor growth. The effects were at least partly reversed by high K^+. Motility was also inhibited by HOQNO and by a series of drugs known to depolarize nerve membranes and block impulse transmission. Colicins E1 and K, which also exert their effects on energy-linked functions of the membrane, are known to inhibit motility of *E. coli* (109). The meaning of these results is not obvious. Faust and Doetsch (103, 104) suggest that a membrane potential may be involved in motility, or that conformational changes induced in the membrane at the base of the flagellum impart to it a rotary motion. These speculations lack clear definition at present, but they may contain the germ of a novel approach to the vexing problem of bacterial motility. It would be of very great importance to study the effects of ionophores in a more sensitive organism and under conditions which lend themselves more readily to interpretation.

Bacteriocins and the Energized State

Throughout this review, I have found it useful to invoke the energized state of the membrane as a link between metabolism and various forms of work, recognizing that this entity may share attributes of a membrane potential, a chemical compound, and of a strained conformational state. Uncouplers are inhibitors which exert their effects at, or close to, this level, and others are found among the bacteriocins. Here we shall consider only colicins of types K, E1, and I which interfere with energy-linked membrane functions (for general reviews see 132, 287).

Colicins K, E1, and I evoke multiple effects in sensitive cells. Synthesis of deoxyribonucleic acid, ribonucleic acid, and protein ceases abruptly; the ATP level falls to about one-third, though respiration continues. Active transport of TMG is blocked, and preexisting pools are discharged, but the permeability barrier in general remains intact. Indeed, the phosphotransferase system is not affected, and the cells can still accumulate α-methylglucoside by vectorial phosphorylation. Motility is inhibited (108, 109, 237). Thus far the effects resemble those of azide and suggest that colicins uncouple oxidative phosphorylation. However, not all the known metabolic effects may be so explained. Fields and Luria (108, 109) found marked shifts in metabolic pathways, with excretion of glucose-6-phosphate, fructose 1,6 diphosphate, and other glycolytic intermediates from the cells. Moreover, hemin-deficient mutants, which should be unable to carry out oxidative phosphorylation, are still colicin-sensitive. Conversely, strict anaerobiosis protects the cells. Similar results have recently been obtained with a bacteriocin from *Serratia* (116).

The suggestion that colicins should not be regarded primarily as uncouplers of oxidative phosphorylation is reinforced by the demonstration that colicin E1 inhibited proline accumulation by membrane vesicles of *E. coli* (31). Since these vesicles do not carry out oxidative phosphorylation, it follows that the primary effect of the antibiotics is likely to be on some aspect of the energized state; the characteristic drop in ATP level is probably a consequence of this primary action, rather than its cause (106).

Confirmation of this suggestion may be found in a paradoxical observation reported by Cramer and Phillips (80): when colicin E1 was added to a suspension of *E. coli* cells containing ANS^-, the fluorescence yield was enhanced. The increased fluorescence of ANS^- paralleled the decrease in the cellular ATP level. It is conceivable that this effect mirrors the collapse of the energized state, and especially that of a membrane potential: like intact mitochondria, respiring *E. coli* may extrude ANS^- by virtue of the electrical potential, interior negative.

Just what the nature of the primary colicin effect may be is presently not known. Colicins bind to highly specific cellular receptors and are believed to remain bound to the receptor sites, external to the permeability barrier, exerting their effects from there. A single, bound-colicin molecule may be lethal. Mutants have been isolated which adsorb colicins but no longer respond to them, and the existence of such colicin-tolerant mutants is often cited as evidence for the existence of a "trans-

mission system," such that the entire membrane responds as a unit to attachment of a single colicin molecule (281, 287, 288). However, a localized lesion or hole in the membrane (386) may equally account for the results. Indeed, Feingold (106) reported the important observation that colicin E1 facilitates diffusion of K^+ across *E. coli* membranes, but not that of protons. Rapid loss of K^+ from cells upon addition of colicin E1 was also described by Hirata et al. (158) and by Wendt (386). Whether K^+ conduction will prove to be the necessary and sufficient cause of the metabolic effects of colicin E1 remains to be seen. It seems, however, that colicins (unlike the classical uncouplers) do not conduct protons and thus make up a distinct class of probes for the nature and physiological role of the energized state.

SUMMARY AND PROSPECT

This essay is an attempt to integrate the fragmentary information on energy transformation by bacterial membranes with the more extensive knowledge available from mitochondria.

It seems now to be generally admitted that mitochondria utilize respiratory energy in a number of fundamentally different ways. ATP synthesis, transhydrogenation, ion transport, and other functions all appear to draw upon a common energy donor whose nature remains elusive. At times it has the qualities of a chemical compound, at others those of a strained conformational state; the transport data can be rationalized only by accepting the existence of a *p*H gradient and electrical potential across the membrane. Efforts to assign a unique nature to this trinity continue, generating a literature whose flavor is at times almost Byzantine.

The main thesis of this article is that in principle, though perhaps not in every detail, the nature and interrelationships of energy-conserving and energy-utilizing reactions in bacteria are the same as those in mitochondria. Not for all regions of the metabolic map (Fig. 1) can this assertion be made with confidence. The case is best for oxidative phosphorylation (and photosynthesis), where a strong argument can be made simply on the grounds of biochemical unity. It is weak indeed for motility, where hard evidence and persuasive analogy are alike lacking. Most intriguing is the emerging inference that bacteria, too, generate gradients of *p*H and of electrical potential across the membrane, and that these are intimately related both to oxidative phosphorylation and to active transport.

The coupling of transport to metabolism poses special challenges. Historically, microbiologists tended to approach transport from the kinetic and genetic side, to the neglect of its metabolic connections. Students of mitochondria achieved a better appreciation of the place of transport in the overall economy of organelles, because they encountered it as a mode of energy utilization alternative to oxidative phosphorylation. It is obvious that these two cultures must interact more than they have done so far, to the mutual enrichment of both. But it should not be taken for granted that models based on mitochondria can be transplanted intact to the bacteria. In nutrient transport, if nowhere else, we can expect to encounter major differences, resulting from the divergent life-styles of intracellular organelle and free-living organism.

Today, when scientists are inundated by what we genially refer to as *The Literature*, a call for more research should not be issued lightly. It is difficult to see how the questions touched upon in this article can be resolved without more experimental data and critical thought. But one has the uneasy feeling that the former is likely to be more plentiful than the latter.

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